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13. ABSTRACT ***(Maximum 200 Words)***

Deregulation of pre-mRNA processing has been linked to malignant transformation and the formation of metastases in breast and other cancers. We are studying the role of a group of proteins that contain domains rich in alternating serine and arginine residues (RS domains) in pre-mRNA processing. RS domain proteins comprise members of the "SR family" and "SR-related" proteins, which function in both constitutive and regulated splicing. The past three years of the Idea Award research has identified the SR-related nuclear matrix protein of 160 kDa (SRm160) and the associated oncoprotein DEK as factors that not only associate with splicing complexes, but which also remain bound to mRNA after splicing. Association of these components with mRNA depends on prior splicing, suggesting that SRm160 and DEK are splicing complex proteins which may function in the coupling of splicing with downstream steps in gene expression, including mRNA 3′-end formation, export, stability and translation. During the final year of research supported by the Idea Award, we have shown that SRm160 participates in mRNA 3′-end formation. Increased levels of SRm160 stimulates 3′-end cleavage, both in vitro and in vivo. Moreover, SRm160 was more efficient in promoting the cleavage of splicing-active than splicing-inactive substrates, indicating that it may function in the coupling of splicing and 3′-end formation. These studies have provided the basis for a more detailed understanding of how gene expression processes communicate. Such an understanding is critical if we are to obtain a complete picture of how genetic alterations in breast cancers and other human diseases might impact on the regulation of gene expression.

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INTRODUCTION

Deregulation of pre-mRNA processing events has been linked to malignant transformation and the formation of metastases in breast and other cancers. For example, specific alternatively spliced forms of the pre-mRNA encoding the cell surface adhesion glycoprotein CD44 have been correlated with invasive tumor formation (Cooper and Dougherty, 1995; Cooper and Mattox, 1997; Taneba and Saya, 1994). Moreover, it has been demonstrated that expression of specific alternative spliced forms of CD44 mRNAs in non-metastatic cell lines result in the transition to full metastatic potential (Günthert *et al.*, 1991). We are interested in understanding the mechanisms underlying the regulation of gene expression at the level of pre-mRNA processing with the long-term aim of identifying how these processes become deregulated in cancers and other disease situations. To this end, much of our work has focused on the machinery that executes constitutive and regulated pre-mRNA splicing, as well as the factors and mechanisms that coordinate splicing with other steps in gene expression, including transcript 3′-end formation and export.

In years 1 and 2 of research supported by the Idea Award, we have characterized pre-mRNA processing factors that contain domains rich in alternating serine and arginine resides (RS domains), as well as proteins that associate with these factors. Proteins that contain RS domains, most often referred to as serine/arginine-repeat (SR) proteins, are known to be important for constitutive and regulated splicing (Blencowe *et al.*, 1999; Graveley, 2000; Smith and Valcarcel, 2000). Our work on two RS domain proteins, Hel-117 (a DEAD-box putative helicase of 117 kDa) and SRm160 (the SR-related nuclear matrix protein of 160 kDa) led to the identification of an associated factor, the oncoprotein DEK. In our work and the work of others, DEK and SRm160 were shown to be associated with splicing complexes and to remain associated with spliced transcripts in manner that is dependent on prior splicing (McGarvey *et al.*, 2000). This interesting property suggested that DEK and SRm160 are possible candidates for mediating downstream steps in gene expression that are influenced by prior splicing. For

example, splicing is important for promoting processes including 3′-end formation, export, turnover (by the nonsense-mediated decay pathway), and translation. Although the functional significance of the association of DEK with splicing complexes and spliced mRNA has not yet been determined, work during the past year supported by the Idea Award has revealed that SRm160 functions in promoting the 3′-end formation of transcripts and suggests an important role for this splicing factor in the coupling of splicing and 3′-cleavage. This work thus contributes to our understanding how steps in gene expression are coordinated. Such an understanding will potentially be important for elucidating how gene expression is be altered in life-threatening diseases including breast cancers. This report describes our work during the past year and its implications. A recent publication in *Mol. Cell. Biol.* that has stemmed from the work is appended to the report. *Note that all figures referred to in the text are contained within the publication.*

BODY

Background to work during year 3 of the idea award research

The processing of pre-mRNA to mature mRNA involves the addition of a 5′ m7GpppG cap, splicing, and 3′-end processing (cleavage and polyadenylation). Although each of these processing steps can occur independently, increasing evidence indicates they are in fact highly integrated and coordinated with each other, as well as with transcription by RNA polymerase II (pol II) (reviewed in: (Hirose and Manley, 2000)). Independent of transcription, formation of a 5′ cap binding complex (CBC) facilitates the recognition of the adjacent, downstream, 5′ splice site, thereby promoting the definition of cap-proximal exons (Izaurralde *et al.*, 1994; Lewis *et al.*, 1996). The CBC can also activate the 3′-end formation of transcripts lacking introns (Flaherty *et al.*, 1997). Splicing of 3′-end-most introns and 3′-end processing can mutually stimulate each other, and interactions between splicing and polyadenylation factors are important for the definition of terminal exons in transcripts (Bauren *et al.*, 1998; Gunderson *et al.*, 1997; Lou *et al.*, 1998; Nesic and Maquat, 1994; Niwa and Berget, 1991; Niwa *et al.*, 1990; Vagner *et al.*, 2000; Wassarman and Steitz, 1993).

Other studies have provided evidence that splicing and 3′-end formation are also highly coordinated with the nuclear retention and export of transcripts. Recognition of the AAUAAA polyadenylation signal by 3′-end cleavage factors is required for transcription termination, as well as for 3′-end formation, and therefore is necessary for the release of pol II transcripts from the nucleus. In addition, intron-containing transcripts are not normally exported because they are retained in the nucleus by interactions with splicing factors (Chang and Sharp, 1989; Custodio *et al.*, 1999; Legrain and Rosbash, 1989; Rutz and Seraphin, 2000). Aside from releasing transcripts from nuclear retention, it has been reported recently that splicing can promote the nuclear export of some transcripts, since the corresponding transcripts derived from intronless constructs were exported less efficiently (Luo and Reed, 1999; Rodrigues *et al.*, 2001; Zhou *et al.*, 2000).

Despite the numerous examples of coupling between different steps in mRNA processing and export, the factors and mechanisms involved are not well understood. Pre-mRNA splicing involves the step-wise association with transcripts of small nuclear ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) and non-snRNP splicing factors, which include SR (serine/arginine-repeat) family and SR-related proteins (reviewed in: (Blencowe *et al.*, 1999; Burge *et al.*, 1999; Fu, 1995; Graveley, 2000; Kramer, 1996; Reed and Palandjian, 1997). Together, these factors form the spliceosome, which executes splicing catalysis. Formation of a poly(A) tail, which is specified by the highly conserved AAUAAA polyadenylation signal and a downstream G- or G/U-rich element, is catalyzed by multi-subunit complexes in two steps: cleavage and then polyadenylation (reviewed in: (Colgan and Manley, 1997; Wahle and Keller, 1996). Several studies have provided evidence that different splicing factors can interact with components of the cleavage/polyadenylation machinery and either stimulate or inhibit polyadenylation (Gunderson *et al.*, 1998; Gunderson *et al.*, 1997; Lou *et al.*, 1998; Lutz *et al.*, 1996; Vagner *et al.*, 2000; Wassarman and Steitz, 1993).

In previous studies, we identified SRm160 (the SR-related nuclear matrix protein of 160kDa), an SR-related protein which functions as a coactivator of both constitutive and exon enhancer-dependent splicing by forming cross-intron interactions with multiple splicing factors bound directly to pre-mRNA (Blencowe *et al.*, 2000; Blencowe *et al.*, 1998; Eldridge *et al.*, 1999). It has been reported recently that SRm160, together with several other factors, including the acute myeloid leukemia-associated protein DEK, the splicing activator RNPS1, the hnRNP-like shuttling protein Y14, and the mRNA shuttling/export factor REF/Aly, bind to mRNAs in a splicing-dependent manner (Kataoka *et al.*, 2000; Le Hir *et al.*, 2000; McGarvey *et al.*, 2000; Zhou *et al.*, 2000). This finding has suggested that SRm160 might participate in one or more steps in mRNA metabolism influenced by prior splicing, including mRNA export.

In the present study, we demonstrate that SRm160 can activate the 3′-end cleavage of transcripts, both *in vitro* and *in vivo*. Consistent with a role in the coupling of splicing and 3′-end formation, SRm160 was found to interact specifically with the cleavage polyadenylation specificity factor (CPSF) and to be more active in promoting the cleavage of splicing-active than of -inactive substrates *in vitro*. Surprisingly, a consequence of over-expression of SRm160 *in vivo* was the uncoupling of the requirement for splicing to promote the 3′-end cleavage and transport of transcripts to the cytoplasm. The results provide evidence for a role for SRm160 in 3′-end processing and demonstrate that the level of this splicing coactivator is critical for maintaining the coordination of pre-mRNA processing events.

RESULTS

In order to investigate whether SRm160 influences steps in mRNA maturation besides splicing, human 293 cells were transfected with plasmids containing different pre-mRNA reporters, with or without an expression plasmid encoding SRm160 fused to an N-terminal FLAG-epitope (pcDNA3-fSRm160). Each pre-mRNA reporter contained an upstream promoter derived from the adenovirus major-late (ML) region and a 3′ late-poly(A) signal from Simian Virus-40 (SV40). Included in all transfections was an RNA pol III VA-RNA reporter plasmid (pSPVA), which serves as an internal control for monitoring the relative transfection efficiency and recovery of RNA between samples. RNA was isolated from both the nuclear and cytoplasmic fractions of the transfected cells and analyzed by RNase protection, in order to determine whether increased levels of SRm160 influences the nuclear-cytoplasmic distribution of transcripts, as well their processing.

Elevated levels of SRm160 *in vivo* results in the cytoplasmic accumulation of unspliced pre-mRNA transcripts

The effect of increased expression of SRm160 was first tested on the processing of a model substrate derived from exons 1 and 2 of human β-globin (hβ-glo) pre-mRNA (Figure 1A). Immunoblotting experiments indicated that, in a typical transfection experiment, the level of fSRm160 was approximately 10 to 20-fold higher than the level of endogenous SRm160 (data not shown). RNA in the nuclear and cytoplasmic fractions was analyzed using the RNase protection probe illustrated in Figure 1A, which spans from 99 bases upstream of the start site of transcription to the 3′-end of exon 2. In the control transfection the hβ-glo transcripts were processed and exported efficiently, resulting in the accumulation of spliced mRNA almost exclusively in the cytoplasmic fraction (Figure 1B, lanes 1,2). However, surprisingly, when fSRm160 was expressed, a high level of unspliced transcripts accumulated in the cytoplasmic fraction, in addition to spliced mRNA (lanes 3,4). The size of the protected product indicates that these unspliced transcripts are correctly initiated. In several independent experiments the level of the

VA RNA did not change significantly following fSRm160 expression (the increase observed between lanes 2 and 4 in Figure 1B is less than 2-fold, and most likely is due to variation in transfection efficiency; refer also to data in Figure 2). Therefore, the effect of increased fSRm160 expression on promoting the cytoplasmic accumulation of unspliced hβ-glo transcripts appears to be specific for pol II transcripts. Thus, increased SRm160 expression appears to prevent or bypass splicing, in addition to uncoupling interactions that normally would retain unspliced transcripts in the nucleus.

We next determined whether expression of fSRm160 influences the processing and nuclear-cytoplasmic distribution of another model transcript, derived from exons 3 and 4 of the *Doublesex* gene of *Drosophila* (dsx pre-mRNA) (Figure 2). The dsx pre-mRNA contains a suboptimal 3′-splice site/polypyrimidine tract and requires an exonic splicing enhancer (ESE) in exon 4 for efficient splicing. Previously, we showed that SRm160 is required for a mammalian ESE, consisting of 6xGAA repeats, to promote the splicing of this substrate *in vitro* (Eldridge *et al.*, 1999). In order to investigate whether SRm160 influences the processing and/or nucleocytoplasmic distribution of this substrate *in vivo*, we next compared the effect of its increased expression on dsx reporters containing or lacking a 6xGAA-repeat ESE in exon 4, designated "dsx$[GAA]_6$" and "dsxΔE", respectively (Figure 2A).

RNA isolated from the nuclear and cytoplasmic fractions of cells transfected with the dsxΔE and dsx$(GAA)_6$ reporters, with or without pcDNA3-fSRm160, was analyzed by RNase protection using the probes illustrated in Figure 2A. The dsx splicing probe spans from -99 bases upstream of the start site of transcription to the 3′-end of exon 4, allowing the detection of unspliced and spliced transcripts, as well as "readaround" transcripts, which can arise by inefficient termination of transcription or incorrect initiation at cryptic promoters. In the absence of fSRm160 expression, insertion of the 6xGAA-ESE in exon 4 resulted in an increase in the efficiency of splicing, similar to its activity observed *in vitro* (eg. see Eldridge et al., 1999) (Figure 2B, compare lanes 1,2 with 5,6). Consistent with numerous previous reports demonstrating the nuclear retention of unprocessed transcripts, the unspliced pre-mRNA and

"readaround" transcripts from both reporter plasmids were detected primarily in the nuclear fractions (compare lanes 1,5 with lanes 2,6) whereas the majority of spliced transcripts from each reporter were detected in the cytoplasmic fractions (compare lanes 2,4,6,8 with lanes 1,3,5,7).

Similar to the results obtained with the hβ-Glo reporter transcripts in Figure 1, fSRm160 expression resulted in a pronounced increase in the level of unspliced pre-mRNA in the cytoplasmic fraction, for both the dsxΔE and dsx$(GAA)_6$ transcripts (compare lanes 2,4 and 6,8). The ratio of cytoplasmic to nuclear unspliced dsxΔE and dsx$(GAA)_6$ pre-mRNA transcripts increased 8-fold and 7-fold, respectively (average values from 4 independent experiments), indicating that the effect is not dependent on the presence of the ESE (see Figure 2D; data not shown). As observed for the hβ-Glo reporter transcripts, the increase in cytoplasmic unspliced pre-mRNA did not coincide with a significant decrease in the level of pre-mRNA in the nuclear fractions, again indicating that fSRm160 expression results predominantly in the cytoplasmic accumulation of a population of unspliced transcripts. Also similar to the results with the hβ-Glo transcripts, increased expression of fSRm160 did not significantly affect the levels of the spliced dsxΔE or dsx$(GAA)_6$ transcripts in the nuclear or cytoplasmic fractions (Figure 1B, compare lanes 3,4 with 1,2 and lanes 7,8 with 5,6). In both cases, it is possible that a population of pre-mRNA transcribed from these reporters is processed and exported prior to fSRm160 reaching levels which result in the cytoplasmic accumulation of unspliced transcripts (see Discussion). Thus, the results demonstrate that elevated levels of SRm160 can promote the cytoplasmic accumulation of distinct pre-mRNA transcripts.

SRm160 promotes the 3′-end cleavage of transcripts *in vivo*

A pre-requisite for the nuclear export of unspliced pre-mRNA is that it is released from nuclear retention factors, which can include transcribing RNA polymerase II (pol II), as well as factors involved in the formation of splicing complexes (Chang and Sharp, 1989; Custodio *et al.*, 1999; Legrain and Rosbash, 1989; Rutz and Seraphin, 2000). Release of transcripts from pol II involves recognition of the AAUAAA poly(A) site by 3′-end cleavage factors, which is important for efficient transcription termination as well

as 3′-end cleavage (Birse *et al.*, 1998; Proudfoot, 2000). One possibility is that increased SRm160 expression allows the release of unspliced transcripts from the nucleus by facilitating 3′-end formation, without the requirement for concomitant splicing. To investigate this, we asked whether fSRm160 expression influences the 3′-end cleavage of the dsx transcripts. Accordingly, the same samples shown in Figure 2B were analyzed with RNase protection probes designed to monitor 3′-end cleavage (Figure 2C; refer to Figure 2A).

Consistent with an important role for splicing in the promotion of 3′-end processing, in the absence of fSRm160 expression, a significant increase in the ratio of cleaved:uncleaved transcripts was detected as a result of insertion of the 6xGAA ESE in exon 4 (Figure 2C, compare lanes 1+2 with lanes 5+6). This increase was 9-fold in the nuclear fraction and 14-fold in the cytoplasmic fraction (data not shown). Moreover, in agreement with an important role for 3′-end processing in facilitating the release of transcripts from the nucleus, essentially all of the transcripts detected in the cytoplasmic fractions were 3′-end cleaved, whereas the uncleaved transcripts were almost entirely detected in the nuclear fractions (Figure 2C, compare lanes 1,2 and 5,6). Significantly, expression of fSRm160 resulted in a further increase (4-fold for dsxΔE and 2-fold for dsx$[GAA]_6$, as determined from averaging values from three independent experiments) in the level of cleaved transcripts in the cytoplasmic fractions, concurring with the increased levels of cytoplasmic unspliced transcripts in these fractions (compare lanes 4,8 with 2,6 in Figures 2B and 2C; Figure 2D and data not shown). The consistently higher level of cleavage-stimulatory activity of fSRm160 observed for the dsxΔE transcripts, compared to the dsx$(GAA)_6$ transcripts, suggests that the level of cleavage promoted by the 6xGAA repeat ESE may already be near saturation. In agreement with the results obtained with the dsx pre-mRNA reporters, and with the results in Figure 1B, elevated levels of SRm160 also resulted in the presence of cleaved, unspliced, hβ-glo pre-mRNA transcripts in the cytoplasmic fractions (data not shown). Thus, elevated levels of SRm160 *in vivo* appear to facilitate the nuclear release of different unspliced transcripts by stimulating 3′-end cleavage.

Promotion of transcript release from the nucleus by SRm160 requires a wild-type polyadenylation signal.

In order to confirm whether SRm160 acts to promote the nuclear release of unspliced transcripts by activating 3′-end formation, we compared its activity on dsx_E transcripts containing either a wild type (AAUAAA, "dsx_E-WT") or a mutant, inactive (AAGAAA, "dsx_E-MT"), SV40-late poly(A) signal (Figure 3A). In agreement with previous experiments indicating that the AAUAAA signal is required for 3′-end cleavage and for RNA pol II to terminate (Birse *et al.*, 1998; Proudfoot, 2000), mutation of the poly(A) signal resulted in the accumulation to high levels in the nuclear fraction of uncleaved, "readaround", transcripts (Figure 3A, lanes 3,5). Although these "readaround" transcripts probably arise as a consequence of the loss of efficient termination of transcription, we cannot exclude the alternative possibility that they also arise through incorrect initiation of transcription. However, in either case, the results demonstrate that fSRm160 expression did not result in the 3′-end cleavage or cytoplasmic accumulation of the dsx_E-MT transcripts. It is noteworthy that co-expression of fSRm160 did not result in a significant change in the level of "readaround" transcripts in the nuclear fraction; the slight decrease observed in Figure 3A is probably due to experimental variation since it was not observed in repeat experiments (lanes 3,5; data not shown). This indicates that the cytoplasmic accumulation of unspliced transcripts following fSRm160 expression is not a consequence of increased levels of transcription from the reporter plasmids. Similar results to the above were observed when comparing wild type and mutant poly(A) signal-derivatives of the dsx$(GAA)_6$ and hβ-glo reporter transcripts (data not shown). These data confirm that activation of 3′-end cleavage by fSRm160 requires the presence of the AAUAAA poly(A) signal, and also demonstrate that increased levels of SRm160 do not promote the nuclear release of transcripts without 3′-end cleavage.

SRm160 can promote the 3′-end cleavage and nuclear release of transcripts independently of splicing

The results so far indicate that elevated levels of SRm160 *in vivo* can promote the 3′-end cleavage and cytoplasmic accumulation of transcripts without the requirement for concomitant splicing. To confirm whether this is the case, we next compared the ability of SRm160 to promote these activities on a splicing-inactive derivative of the dsx___reporter, which contains a deletion in the 5′ splice site (dsx_5′ss) (Figure 3B). RNase protection analysis with a probe spanning the intron and exon sequences of this transcript confirmed that it was not spliced (data not shown). Similar to the results observed for the unspliced dsx_E transcripts, RNase protection analysis using the dsx_E 3′-end cleavage probe (refer to Figure 2A) revealed that elevated levels of SRm160 resulted in a pronounced increase in the level of cleavage and cytoplasmic accumulation of the dsx_5′ss transcript (Figure 3B and data not shown). This confirms that the presence of a functional intron and splicing is not required in order for SRm160 to promote 3′-end cleavage and nuclear release of transcripts. As will be expanded on below, although excess levels of SRm160 can result in the activation of 3′-end cleavage independently of splicing, it may normally only promote 3′-end cleavage when coupled to splicing.

Specificity of the cleavage-stimulatory and "export" activities of SRm160

In order to investigate the specificity of SRm160 in promoting 3′-end cleavage and cytoplasmic accumulation of transcripts *in vivo*, its activity was initially compared alongside the SRm160-interacting factor DEK, which, like SRm160, associates with splicing complexes and forms a component of a splicing-dependent exon junction complex (see Introduction). Transient transfection and RNAse protection assays were performed as described above using the dsx$(GAA)_6$ pre-mRNA reporter, together with an expression plasmid encoding DEK (Figure 4A). Although DEK was efficiently expressed, unlike SRm160, it did not result in a significant level of stimulation of 3′-end cleavage or accumulation of pre-mRNA in the cytoplasm (Figure 4A, compare lanes 3,4 with 1,2 and 5,6; data not shown).

Several FLAG epitope-tagged deletion derivatives of SRm160 were also tested for their ability to stimulate cleavage and cytoplasmic accumulation of transcripts. Although these deletion proteins were

expressed as efficiently as wild-type fSRm160, the majority did not promote 3′-end cleavage or cytoplasmic-pre-mRNA accumulation of dsx reporter transcripts (data not shown). However, interpretation of the results was complicated since many of the inactive deletion derivatives did not localize in the same manner as wild-type SRm160, raising the possibility that their lack of function could be a consequence of mislocalization. However, one of the deletions (fSRm160_N1), which lacks residues 1-155 corresponding to the highly conserved N-terminal domain of SRm160, localized in the same nuclear speckled pattern as wild-type SRm160, yet was not active in promoting the 3′-end cleavage and cytoplasmic accumulation of transcripts. This is demonstrated in the RNase protection analysis of dsx_E pre-mRNA splicing and cleavage shown in Figure 4C (compare lanes 3,4 with 1,2 and 5,6), in which cleavage was analyzed with the same probe as described in Figure 2A, and splicing was analyzed using the probe illustrated in Figure 4B. This latter probe spans from the start of exon 3 to the middle of the dsx intron. It is noteworthy that, while inactive, SRm160_N1 retains the RS domain and other repeat motifs that are probably important for its correct localization and interactions with other pre-mRNA processing factors. This result indicates that intact SRm160 is important for stimulation of 3′-end cleavage and cytoplasmic accumulation of pre-mRNA, and that both of these activities depend on the presence of the conserved N-terminal domain of the protein.

SRm160 interacts with the cleavage polyadenylation specificity factor (CPSF)

The activity of SRm160 in promoting 3′-end processing in the experiments described above could result from one or more indirect effects arising from its increased expression levels, or the more interesting possibility that it participates more directly in promoting 3′-end cleavage. In order to distinguish between these possibilities, we next asked whether SRm160 associates with one or more components of the 3′-end cleavage machinery.

Immunoprecipitates were collected from HeLa nuclear extract using a monoclonal antibody specific for SRm160 (mAb-B1C8) (Blencowe *et al.*, 1994; Wan *et al.*, 1994). Monoclonal antibody-B1C8 has

previously been shown to immunoprecipitate SRm160 in complexes containing SR-family proteins, the SR-related proteins hTra2-beta and Hel117, and the oncoprotein DEK (Eldridge *et al.*, 1999; McGarvey *et al.*, 2000) (our unpublished observations). The mAb-B1C8 immunoprecipitates were immunoblotted with available antisera specific for 3′-end processing factors, including CstF-77 (Cleavage Stimulation Factor 77 kDa subunit), CPSF-160 (Cleavage-Polyadenylation-Specificity-Factor 160 kDa subunit) and PAP (Poly(A) Polymerase). Although all three of these antibodies detected proteins of the expected sizes in HeLa nuclear extract, only CPSF-160 was significantly enriched in the mAb-B1C8 immunoprecipitates (Figure 5A, lane 4; data not shown). Approximately 2% of CPSF-160 in the nuclear extract was immunoprecipitated with mAb-B1C8, indicating that only a low level of this cleavage factor interacts with SRm160, or else that this interaction is unstable during immunoprecipitation. Nevertheless, the interaction was specific since CPSF-160 was not substantially co-immunoprecipitated with excess amounts of non-specific control antibodies (lanes 3,5 and data not shown). Moreover, CPSF-160 was still co-immunoprecipitated by mAb-B1C8 after extensive pre-treatment of the nuclear extract with RNase (compare lanes 1 and 2 in Figure 5B), indicating that it probably associates with SRm160 through protein-protein interactions (Figure 5A, compare lanes 4 and 6).

In order to confirm whether SRm160 and CPSF associate specifically, a reciprocal immunoprecipitation experiment was performed in which immunoprecipitates were collected from RNase pre-treated HeLa nuclear extract using antisera specific for the 73 kDa subunit of CPSF (CPSF-73), PAP and CstF-77, and then immunoblotted with mAb-B1C8 (Figure 5C). All of these antibodies immunoprecipitate their target proteins efficiently, whereas the anti-CPSF-160 antibody used above does not and therefore was not included in the analysis (data not shown). The anti-CPSF-73 antibody resulted in a significant level of enrichment of SRm160 (lane 4), whereas little or no co-immunoprecipitation was observed, above the background level with a control antibody, with the anti-PAP or anti-CstF-77 antibodies (compare lane 3 with lanes 5 and 6, respectively). Approximately 2-3% of SRm160 was co-immunoprecipitated with

anti-CPSF-73, again indicating that a relatively low level of SRm160 and CPSF associate specifically in HeLa nuclear extract.

SRm160 stimulates 3′-end cleavage *in vitro*

The activity of SRm160 in promoting 3′-end processing was next investigated by determining whether it can activate 3′-end cleavage of different RNA substrates with functional polyadenylation sites *in vitro* (Figure 5). To first determine whether SRm160 can promote cleavage independent of splicing, highly purified, baculovirus-expressed SRm160 (bSRm160) (Figure 5A, refer to Blencowe et al., 2000), was added to 3′-end cleavage reactions containing a substrate derived from the 3′-half of exon 4 of the dsx_E pre-mRNA, fused to either a wild-type (AAUAAA; "dsx_-p(A)-WT") or mutant (AAGAAA; "dsx_-p(A)-MT") late-poly(A) signal from SV40 (Figure 5B). Significantly, increasing amounts of bSRm160 stimulated, up to approximately 3-fold, cleavage of the dsx_-p(A)-WT but not the dsx_-p(A)-MT substrate (Figure 5B, compare lanes 1-3 with lanes 4-6; note that although these substrates differ only by the U_G substitution in the AAUAAA sequence, they migrate differently due to a structural difference conferred by this substitution). The results demonstrate that SRm160, similar to its activity *in vivo*, can promote 3′-end cleavage *in vitro*. Moreover, its cleavage-stimulatory activity does not depend on the presence of active splice sites.

Specificity of the cleavage-stimulatory activity of SRm160 *in vitro*

In order to investigate the specificity of the 3′-end cleavage-stimulatory function of SRm160 *in vitro*, we compared its activity with two different baculovirus-expressed SR proteins, SRp30c and SRp40 (Figure 5C). Both of these proteins were purified to near homogeneity and were active in splicing reconstitution assays performed in HeLa S100 reactions (data not shown). Titration of equal amounts of these proteins as SRm160 in 3′-cleavage reactions containing the dsx_-p(A)-WT substrate did not significantly influence the ratio of cleaved:uncleaved substrate, whereas approximately a 4-fold increase was consistently observed for SRm160 (Figure 5C; data not shown). These differences in cleavage-

stimulatory activity are not a consequence of differences in the lengths of the RS domains of these proteins since SRp40 contains a higher number of consecutive SR/RS repeats compared to SRm160, whereas SRp30c contains fewer repeats. Moreover, we have observed that multiple domains of SRm160 other than the RS-rich regions of the protein are important for promoting 3′-end cleavage *in vivo* (see Figure 4C; our unpublished observations). Thus, the results indicate that the activity of SRm160 in stimulating 3′-end processing *in vitro* is not a general feature of RS domain proteins and, moreover, does not reflect the length of the RS domains of these splicing factors.

SRm160 augments the splicing-dependent enhancement of 3′-end cleavage *in vitro*

The experiments presented so far demonstrate a cleavage-stimulatory activity of SRm160 that can function independently of splicing. However, at endogenous levels, SRm160 normally only associates stably with transcripts in the context of functional splicing complexes ((Blencowe *et al.*, 1998; Eldridge *et al.*, 1999), and may therefore provide an important role in 3′-end formation coupled to splicing. To investigate whether the 3′-end cleavage-stimulatory property of SRm160 is augmented by the formation of functional splicing complexes, we next compared its influence on the 3′-end cleavage of splicing-active and -inactive derivatives of an Adenovirus-derived pre-mRNA substrate (MXSVL) (Niwa *et al.*, 1990), each containing the late-poly(A) signal from SV40 (Figure 6D,E). Importantly, bSRm160 was added to splicing/cleavage reactions containing these substrates at levels that were stimulatory to splicing, thus allowing its activity in 3′-end cleavage to be assessed in the context of productive splicing complex formation.

Splicing/cleavage reactions containing wild-type MXSVL (WT) (Figure 6D), or a splicing-inactive derivative lacking a 5′ splice site MXSVL (_5′) (Figure 6E), were incubated with or without the ATP analog 5′-cordycepin triphosphate, in order to distinguish the 3′-end cleaved products. In the presence of 5′-cordycepin triphosphate, polyadenylation is prevented, resulting in the accumulation of 3′-end cleaved products. In the absence of 5′-cordycepin triphosphate, the 3′-end cleaved products are polyadenylated

and migrate as a smear in the upper region of the gel (Figures 5D,E, compare lanes 1-3 with lanes 4-6). The different products detected in the reactions with the MXSVL substrates correspond to those previously characterized in detail by Niwa and Berget (Niwa and Berget, 1991; Niwa *et al.*, 1990) and were assigned accordingly.

Quantification of the reaction intermediates and products revealed that, at the highest level of bSRm160 addition (220ng) to reactions containing the WT-MXSVL substrate, only minor (< 2-fold) changes in the level of unspliced/cleaved pre-mRNA and spliced/uncleaved transcripts were observed. However, there was a 7-fold increase in the level of spliced/cleaved mRNA (Figure 6D, compare lanes 1-3; data not shown). In contrast, addition of 220ng of bSRm160 to reactions containing the Δ5′-MXSVL substrate resulted in an approximately 3-fold increase in the level of cleavage of this pre-mRNA (Figure 6E, compare lanes 1-3). Similarly, addition of 220ng of bSRm160 to a MXSVL substrate lacking a functional 3′ splice site also resulted in an approximate 3-fold increase in 3′-end cleavage (data not shown). Thus, in agreement with the results obtained with the dsx_-p(A) substrate (Figure 6B), SRm160 can promote 3′-end cleavage of the MXSVL substrate independently of splicing. However, the presence of functional splice sites and splicing of the MXSVL pre-mRNA appears to augment the activity of bSRm160 in promoting 3′-end cleavage. These results, taken together with the data in Figure 5 indicating that SRm160 can associate with CPSF, provide evidence that SRm160 participates in the coupling of splicing and 3′-end processing.

DISCUSSION

The results described in this report provide new information on the coordination of splicing with 3′-end formation and the nuclear-cytoplasmic transport of transcripts. SRm160 (the SR-related nuclear matrix protein of 160 kDa), which previously was shown to promote both constitutive and exon enhancer-dependent splicing, was found to stimulate 3′-end cleavage. At elevated levels *in vivo*, SRm160 increased the levels of distinct pre-mRNAs, consistent with previous evidence that its specific ratio to other splicing factors is critical for optimal splicing. Under these conditions, SRm160 activated the 3′-end cleavage of the unspliced transcripts, thereby circumventing the normal requirement for splicing to promote the cleavage of these substrates. A consequence of the activation of pre-mRNA 3′-end cleavage by SRm160 was the accumulation of the unspliced transcripts in the cytoplasm. Thus, elevated levels of SRm160 appear to bypass the normal requirement of splicing for the nuclear release of transcripts. Consistent with a more direct role in activating the 3′-end formation of transcripts, SRm160 was found to associate with the Cleavage Polyadenylation Specificity Factor (CPSF) and to promote 3′-end cleavage *in vitro*. Importantly, although SRm160 promoted 3′-end cleavage independently of splicing, its cleavage-stimulatory activity was enhanced by the concomitant splicing of a transcript *in vitro*. In summary, the results demonstrate a role for SRm160 in 3′-end cleavage and provide evidence that the level of this splicing coactivator is not only important for optimal splicing, but also for the coordination of splicing with 3′-end formation and nuclear retention of incompletely processed transcripts.

Coupling of splicing and 3′-end formation

The mechanism(s) by which the splicing and 3′-end processing machineries communicate with each other is not well understood. Several reports have provided evidence for an important role for U1 snRNP components in 3′-end formation. Antibodies to Sm and U1 snRNP proteins were shown to inhibit polyadenylation *in vitro* (Moore and Sharp, 1984). Subsequently, it was shown that U1 snRNA cross-links to polyadenylation efficiency elements upstream of the poly(A) site, and that the efficiency of this

cross-linking correlates with the efficiency of 3′-end formation (Wassarman and Steitz, 1993). It was also reported that the U1 snRNP-A protein can interact with CPSF, and promote increased polyadenylation *in vitro* (Lutz *et al.*, 1996). Other studies indicated that the binding of U1 snRNP and the SR family protein SRp20 to an intronic splicing enhancer sequence within the alternatively spliced, calcitonin/calcitonin gene-related-peptide pre-mRNA, correlates with increased 3′-end processing at an adjacent poly(A) site (Lou *et al.*, 1998). In other contexts, both U1 snRNP-A and -70 kDa proteins inhibit polyadenylation by interacting with poly(A) polymerase, whereas an interaction between poly(A) polymerase and the U2AF-65 kDa subunit has been shown to increase splicing efficiency (Gunderson *et al.*, 1998; Gunderson *et al.*, 1997; Vagner *et al.*, 2000).

The association of SRm160 with pre-mRNA splicing substrates *in vitro* is normally strongly dependent on U1 snRNP and is further promoted by SR family proteins and U2 snRNP. It is possible that the activity of one or more U1 snRNP components promoting 3′-end formation described above could involve interactions mediated by SRm160. Although in the present study CPSF-160 was found to associate with SRm160, this interaction could be bridged by one or more intermediary factors. For example, in previous studies, we found that SRm160 interacts with several SR family and SR-related proteins (Blencowe *et al.*, 1998; Eldridge *et al.*, 1999) (unpublished observations). Moreover, a recent report indicates that CPSF interacts indirectly with the cleavage factor CF I_m (Cleavage Factor I) (de Vries *et al.*, 2000), the 68 kDa subunit of which, like SRm160, is an SR-related protein (Ruegsegger *et al.*, 1998). Since the alternating arginine/serine (RS) domains of SR family and SR-related proteins interact and are important for the formation of protein-protein interactions, it is possible that the RS domain of CF I-68 could interact with one or more SR/SR-related splicing factors, including SRm160.

A possible role for RS domain proteins, other than SRm160, in modulating 3′-end cleavage is supported indirectly by previous observations. A 22-nucleotide element from the histone *H2a* gene, which promotes both the 3′-end formation and export of transcripts, binds to the SR family proteins 9G8 and SRp20

(Huang and Steitz, 2001). Although antibodies to these SR family proteins inhibited mRNA export, it was not determined whether they also interfered with cleavage. Elevated expression of the SR family protein SC35 and the SR-related/helicase-like protein HRH1(hPRP22), like SRm160, can result in the inhibition of splicing and cytoplasmic accumulation of pre-mRNA, although the mechanism(s) underlying these effects were not investigated (Ono *et al.*, 1994; Wang and Manley, 1995). Our results suggest that these factors could facilitate the nuclear release of transcripts by stimulating cleavage, perhaps in association with SRm160, or through functionally-related yet distinct interactions. However, it is important to note that the effects we have observed for SRm160 are not general properties of RS domain proteins. Expression of elevated levels of the SR family protein ASF/SF2 does not result in 3′-end cleavage or cytoplasmic accumulation of transcripts (Wang and Manley, 1995) (S.M. and B.J.B., unpublished observations), and elevated expression of U1-70K inhibits splicing but prevents the export of transcripts (Romac and Keene, 1995). Moreover, in the present study, we have shown that, unlike SRm160, the SR family proteins SRp30c and SRp40 do not significantly influence 3′-end cleavage *in vitro*. Thus, SRm160 may be representative of a specific subset of SR proteins that can influence 3′-end processing and the nuclear-cytoplasmic distribution of transcripts.

SRm160 and the nuclear export of transcripts

In order for RNA pol II transcripts to be efficiently exported from the nucleus, they must first be released from nuclear retention factors. Recognition of splicing signals by factors that function early in the formation of splicing complexes is important for the retention of unspliced pre-mRNA in the nucleus (Chang and Sharp, 1989; Custodio *et al.*, 1999; Legrain and Rosbash, 1989; Rutz and Seraphin, 2000). Elevated expression levels of SRm160 resulted in the accumulation of unspliced transcripts in the cytoplasm, suggesting that it can either prevent, or else bypass, splicing complex formation. Moreover, the accumulation of unspliced transcripts in the cytoplasm suggests that excess SRm160 might result in the bypass of processes that normally degrade unprocessed RNA in the nucleus. In conjunction with these roles, it is also possible that excess SRm160 prematurely activates an mRNA export pathway,

allowing efficient export of unprocessed transcripts (see below). However, regardless of the mechanism(s) by which increased SRm160 expression results in the accumulation of unspliced transcripts in the cytoplasm, the present results demonstrate that SRm160 can facilitate the release of transcripts from nuclear retention by stimulating 3′-end formation. In particular, increased levels of SRm160 did not relieve the nuclear retention of unspliced transcripts containing a mutant poly(A) site, indicating that its ability to promote the nuclear release of unspliced transcripts is, at least in part, a consequence of its ability to stimulate the 3′-cleavage of these transcripts.

It is important to note that, although excess levels of SRm160 could stimulate 3′-end formation independently of splicing, levels of SRm160 that were not inhibitory to splicing were more efficient in stimulating the 3′-end cleavage of splicing-active than -inactive pre-mRNAs *in vitro*. Taken together with previous results demonstrating a requirement for U1 snRNP and SR family proteins for the association of SRm160 with pre-mRNA (Blencowe *et al.*, 1998; Eldridge *et al.*, 1999), these findings suggest that SRm160 normally promotes 3′-end formation during the formation of productive splicing complexes. Furthermore, recent work has shown that SRm160 forms part of a splicing-dependent complex, 20-24 nucleotides upstream of exon-exon junctions, that contains several factors including DEK, Y14, RNPS1, and the mRNA export factor REF (Le Hir *et al.*, 2000; Zhou *et al.*, 2000). It is therefore possible that the association of SRm160 with this complex at 3′-most exon-exon junctions might facilitate mRNA export by forging interactions with one or more export factors, including REF, as well as by promoting 3′-end cleavage. Similarly, increased expression of SRm160 could result in the recruitment of export factors to unspliced transcripts, thereby facilitating their nuclear release at a step in addition to 3′-end formation.

KEY RESEARCH ACCOMPLISHMENTS DURING YEAR 3

- **Identification of a role for SRm160 in the 3′-end formation of transcript**

- **Demonstration that the level of SRm160 is critical for the proper coordination of pre-mRNA processing.**

- **Demonstration of interactions between SRm160 and 3′-end cleavage components**

- **Demonstration that efficient 3′-end formation is critical for transcript export**

REPORTABLE OUTCOMES

Publications 2000-2001

Blencowe, B.J., Bauren, G., Eldridge, A.E., Issner, R. Nickerson, J.A., Rosonina, E. and Sharp, P.A. (2000). The SRm160/300 splicing coactivator subunits. *RNA*. 6: 111-120.

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Longman, D., McGarvey, T., McCracken, S., Johnstone, I.L., Blencowe, B.J.** and Caceres, J.F**. 2001. Multiple interactions between SRm160 and SR family proteins in enhancer-dependent splicing and development of *Caenorhabditis elegans*. 11, 1923-1933.

McCracken, S., Lambermon, M., and Blencowe, B.J. (2002). The SRm160 splicing coactivator promotes transcript 3′-end formation. *Mol. Cell. Biol.* 22. 148-160

Invited Talks and Meetings 2000-2001

* Functions of Novel Nuclear Matrix Proteins in the Processing of pre-mRNA". Dept. of Molecular Biology and Genetics. Guelph University. 1/00

* McGarvey, T., Rosonina, E., McCracken, S., Li, Q., Nickerson, J.A, Awrey, D., Greenblatt, J. Grosveld, G. and Blencowe, B.J. The acute myeloid leukemia-associated protein DEK is a component of a spliceosome-derived mRNP. RNA Society Meeting 2000. Madison, Wisconsin.

** Regulation of alternative splicing in mammalian cells. Invited seminar at Rosetta Impharmatics, Kirkland, WA. 3/2001

* Biochemical and genetic interactions between the SRm160 splicing coactivator and SR family proteins. Dása Longman, Tim McGarvey, Andrew MacMillan, Iain L. Johnstone, Benjamin J. Blencowe and Javier F. Cáceres. RNA Society Meeting 2001. Bamff, Alberta.

* The SRm160 splicing coactivator promotes the 3′-end processing and nuclear export of transcripts. Susan McCracken, Mark Lambermon, Elisa Izaurralde and Benjamin J. Blencowe. RNA Society Meeting 2001. Bamff, Alberta.

The PWI motif: A novel RNA binding domain in SRm160 and other splicing factors. B.R. Szymczyna, J. Bowman, A. Pineda-Lucena, E. Rosonina, B.J. Blencowe, C.H. Arrowsmith. RNA Society Meeting 2001. Bamff, Alberta.

Non-consensus heptapeptide repeats within the CTD of RNA polymerase II are important for the efficient inclusion of an alternatively spliced exon E. Rosonina and B.J. Blencowe. RNA Society Meeting 2001. Bamff, Alberta.

A genome-wide survey of SR-repeat proteins. L. Boucher, C. Ouzounis and B.J. Blencowe. RNA Society Meeting 2001. Bamff, Alberta.

CONLUSIONS

The results described in this report demonstrate a role for the SRm160 splicing coactivator in mRNA 3′-end formation, and provide evidence that it couples splicing to 3′-end cleavage. Our work during the past three years of the Idea Award research has thus provided important new insights into how splicing is regulated as well as how it is coordinated with other steps in gene expression. Such information is relevant to understanding the full impact of genetic changes which lead to malignant transformation in breast and other cancers. For example, splicing defects that have been identified previously in breast and other cancers could also alter 3′-end formation and/or the efficiency of transcript export or turnover, particularly if they affect the splicing of 3′-most introns. Although this possibility has not been examined, the recent accumulation of evidence for coupling of different steps in gene expression suggests that disease associated alterations in splicing cannot be considered in isolation, but that their more indirect impact on coupled steps in expression must be examined as well. Our work in the present study on SRm160 provides the foundation for understanding how splicing is coupled to other steps in

gene expression, particularly 3′-end formation, and therefore will facilitate an understanding of mechanisms of normal gene expression as well as how these mechanism might go awry in cancer cells.

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APPENDIX 1

(Publication Attached- contains the figures referred to in the Body of the Report)

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SRm160 Splicing Coactivator Promotes Transcript 3′-End Cleavage

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Individual steps in the processing of pre-mRNA, including 5′-end cap formation, splicing, and 3′-end processing (cleavage and polyadenylation) are highly integrated and can influence one another. In addition, prior splicing can influence downstream steps in gene expression, including export of mRNA from the nucleus. However, the factors and mechanisms coordinating these steps in the maturation of pre-mRNA transcripts are not well understood. In the present study we demonstrate that SRm160 (for serine/arginine repeat-related nuclear matrix protein of 160 kDa), a coactivator of constitutive and exon enhancer-dependent splicing, participates in 3′-end formation. Increased levels of SRm160 promoted the 3′-end cleavage of transcripts both in vivo and in vitro. Remarkably, at high levels in vivo SRm160 activated the 3′-end cleavage and cytoplasmic accumulation of unspliced pre-mRNAs, thereby uncoupling the requirement for splicing to promote the 3′-end formation and nuclear release of these transcripts. Consistent with a role in 3′-end formation coupled to splicing, SRm160 was found to associate specifically with the cleavage polyadenylation specificity factor and to stimulate the 3′-end cleavage of splicing-active pre-mRNAs more efficiently than that of splicing-inactive pre-mRNAs in vitro. The results provide evidence for a role for SRm160 in mRNA 3′-end formation and suggest that the level of this splicing coactivator is important for the proper coordination of pre-mRNA processing events.

The processing of pre-mRNA to mature mRNA involves the adding of a 5′ m7GpppG cap, splicing, and 3′-end processing (cleavage and polyadenylation). Although each of these processing steps can occur independently, increasing evidence indicates they are, in fact, highly integrated and coordinated with each other as well as with transcription by RNA polymerase II (pol II) (reviewed in reference 18). Independent of transcription, formation of a 5′ cap binding complex facilitates the recognition of the adjacent, downstream, 5′ splice site, thereby promoting the definition of cap-proximal exons (21, 26). The cap binding complex can also activate the 3′-end formation of transcripts lacking introns (13). Splicing of 3′-end-most introns and 3′-end processing can stimulate each other, and interactions between splicing and polyadenylation factors are important for the definition of terminal exons in transcripts (1, 17, 27, 33–35, 43, 47).

Other studies have provided evidence that splicing and 3′-end formation are also highly coordinated with the nuclear retention and export of transcripts. Recognition of the AAU AAA polyadenylation signal by 3′-end cleavage factors is required for transcription termination as well as for 3′-end formation and therefore is necessary for the release of pol II transcripts from the nucleus. In addition, intron-containing transcripts are not normally exported because they are retained in the nucleus by interactions with splicing factors (8, 10, 24, 42). Aside from releasing transcripts from nuclear retention, it has been reported recently that splicing can promote the nuclear export of some transcripts, since the corresponding transcripts derived from intronless constructs were exported less efficiently (28, 39, 49).

Despite the numerous examples of coupling between different steps in mRNA processing and export, the factors and mechanisms involved are not well understood. Pre-mRNA splicing involves the step-wise association with transcripts of snRNPs, including U1, U2, U4/U6, and U5 snRNPs, and non-snRNP splicing factors, which include SR (serine/arginine repeat) family and SR-related proteins (reviewed in references 4, 7, 14, 15, 23, and 38). Together these factors form the spliceosome, which executes splicing catalysis. Formation of a poly(A) tail, which is specified by the highly conserved AAUAAA polyadenylation signal and a downstream G- or G/U-rich element, is catalyzed by multisubunit complexes in two steps: cleavage and then polyadenylation (reviewed in references 9 and 44). Several studies have provided evidence that different splicing factors can interact with components of the cleavage and polyadenylation machinery and either stimulate or inhibit polyadenylation (16, 17, 27, 29, 43, 47).

In previous studies we and others identified SRm160 (the SR-related nuclear matrix protein of 160 kDa), an SR-related protein which functions as a coactivator of both constitutive and exon enhancer-dependent splicing by forming cross-intron interactions with multiple splicing factors bound directly to pre-mRNA (3, 5, 12). It has been reported recently that SRm160, together with several other factors, including the acute myeloid leukemia-associated protein DEK, the splicing activator RNPS1, the hnRNP-like shuttling protein Y14, and the mRNA shuttling and export factor REF/Aly, bind to mRNAs in a splicing-dependent manner (22, 25, 31, 49). This finding has suggested that SRm160 might participate in one or more steps in mRNA metabolism influenced by prior splicing, including mRNA export.

In the present study we demonstrate that SRm160 can acti-

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vate the 3′-end cleavage of transcripts both in vitro and in vivo. Consistent with a role in the coupling of splicing and 3′-end formation, SRm160 was found to interact specifically with the cleavage polyadenylation specificity factor (CPSF) and to be more active in promoting the cleavage of splicing-active substrates than of splicing-inactive substrates in vitro. Surprisingly, a consequence of overexpression of SRm160 in vivo was the uncoupling of the requirement for splicing to promote the 3′-end cleavage and transport of transcripts to the cytoplasm. The results provide evidence for a role for SRm160 in 3′-end processing and demonstrate that the level of this splicing coactivator is critical for maintaining the coordination of pre-mRNA processing events.

MATERIALS AND METHODS

Plasmids. Details of reporter and RNase protection-probe plasmids can be found at http://www.utoronto.ca/intron/supp_info. The predicted sizes for the various RNase protection products, generated by using the probes shown in Fig. 1A, 2A, and 4B, can also be found at the above website. The pol III-adenovirus-associated (VA) reporter (pSPVA) and the corresponding RNase protection probe plasmid have been described previously (48).

Transfections. Human 293 cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum to a density of 2×10^6 cells per 150-mm-diameter plate prior to transient transfection using calcium phosphate precipitation. Transfections were typically performed with 5 μg of reporter plasmid, 5 to 10 μg of pcDNA3-Flag-SRm160, or the corresponding empty vector. Each transfection contained 0.5 μg of the pol III-VA RNA reporter as an internal control for transfection efficiency as well as for recovery of RNA in the nuclear and cytoplasmic fractions. Following transfection the culture medium was changed after 16 h and the cells were harvested after 40 h.

RNA preparation and analysis. Nuclear and cytoplasmic RNA fractions from the transfected cells were prepared as previously described (19, 30). In each experiment 10% (2 to 5 μg) of the total amount of RNA recovered from each fraction was analyzed. Proportional amounts of RNA from the nuclear and cytoplasmic fractions were analyzed. RNase protections were performed using gel-purified probes, as described by Yankulov et al. (48), except that incubations with RNase were performed for 1 h. RNase protection products were quantified by using a Molecular Dynamics PhosphorImager and ImageQuant software. Each RNA species was quantified, following background subtraction and normalization for VA signal and U content of the protected probe fragment. In order to compare the relative amounts of RNAs from different experiments, the corrected signals were further normalized by being adjusted to the level of spliced or cleaved nuclear RNA (value set to 100). For quantification of splicing of the dsx pre-mRNA reporters exon 3 was measured, since it has a higher A content and was easier to visualize than exon 4. Experimental values were averaged from multiple experiments ($n = 3$ or 4), and standard deviations were calculated by using Microsoft Excel.

Antibodies and immunoprecipitation. Immunoprecipitation of SRm160-containing complexes was performed as described by Eldridge et al. (12). Immunoprecipitation was performed by using a murine monoclonal antibody (MAb) specific for SRm160 (MAb-B1C8) (6, 45) and a control rabbit anti-mouse antibody. Immunoprecipitation with antibodies to CPSF-73, poly(A) polymerase (PAP), and the 77-kDa subunit of human cleavage stimulation factor (CstF-77) was performed using 10 μg of affinity-purified antibody (kind gift of D. Bentley) cross-linked to protein A-Sepharose. Prior to immunoprecipitation, nuclear extract was preincubated for 15 min at 30°C under splicing conditions, with or without the addition of an RNase cocktail (7 ng/μl; Boehringer), and in the presence of phosphatase inhibitors (potassium fluoride, sodium pyrophosphate, and sodium β-glycerophosphate). RNA isolated (by phenol-chloroform extraction and precipitation) from aliquots of the nuclear extracts incubated with or without RNase was separated on a 10% denaturing polyacrylamide gel and detected by ethidium bromide staining.

In vitro transcription and in vitro cleavage reactions. Transcription of RNase protection probes and substrates for in vitro splicing assays was performed essentially as described previously (5). Details of the substrates used for analyzing 3′-end cleavage in vitro can be found at http://www.utoronto.ca/intron/supp_info. The MXSVL derivatives were transcribed by using SP6 RNA polymerase from *Dra*I-linearized pMXSVL-WT and pMXSVL-Δ5′ templates, respectively (35). The cleavage reactions were performed essentially as described previously by Niwa et al. (35), except that the reaction mixtures were preincubated on ice for 10 min with or without baculovirus-expressed SRm160 (bSRm160) and then were preincubated for 2 min at 30°C. Substrates were then added and the reaction mixtures were incubated for 1 h at 30°C. Each reaction mixture contained 2.2 μl of nuclear extract and 1.5 mM $MgCl_2$ (final concentration) and was incubated with or without 5′-cordycepin triphosphate. Purified bSRm160 was added to the reaction mixtures in the amounts described in the legend to Fig. 5.

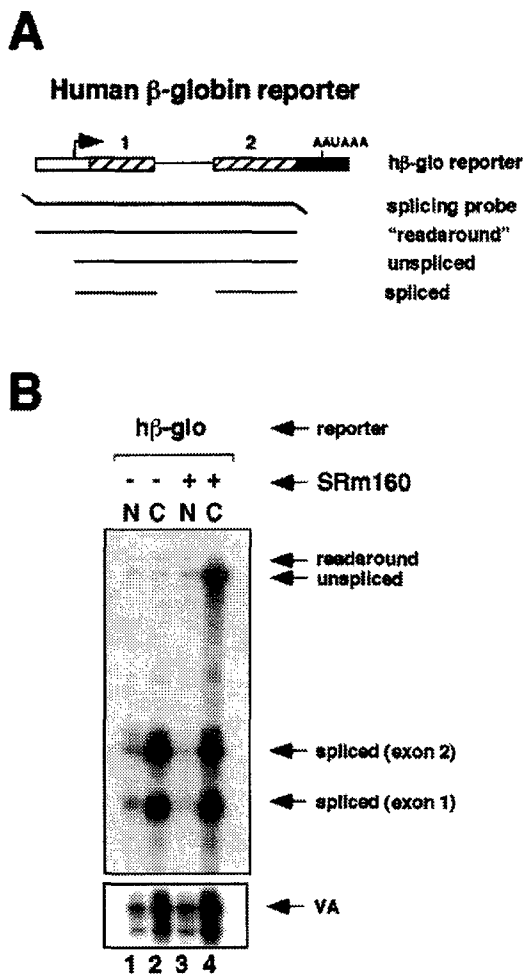

FIG. 1. Expression of high levels of SRm160 in vivo results in the cytoplasmic accumulation of unspliced RNA. (A) Schematic representation of the RNase protection probe used to analyze splicing of transcripts from a reporter consisting of hβ-glo exons 1 and 2 with the intervening intron. The predicted RNase protection products are shown below the probe (for sizes refer to Materials and Methods and supplementary information located at http://www.utoronto.ca/intron/supp_info). (B) Human 293 cells were transiently transfected with the hβ-glo pre-mRNA reporter and a pol III reporter (pSPVA) as an internal control (lanes 1 to 4) together with a control, empty expression vector (pcDNA3-Flag) (lanes 1 and 2) or an expression vector for Flag epitope-tagged SRm160 (pcDNA3-fSRm160) (lanes 3 and 4). Proportional amounts of RNA isolated from the nuclear (N) and cytoplasmic (C) fractions were analyzed by RNase protection using the probe illustrated in panel A.

RESULTS

In order to investigate whether SRm160 influences steps in mRNA maturation besides splicing, human 293 cells were trans-

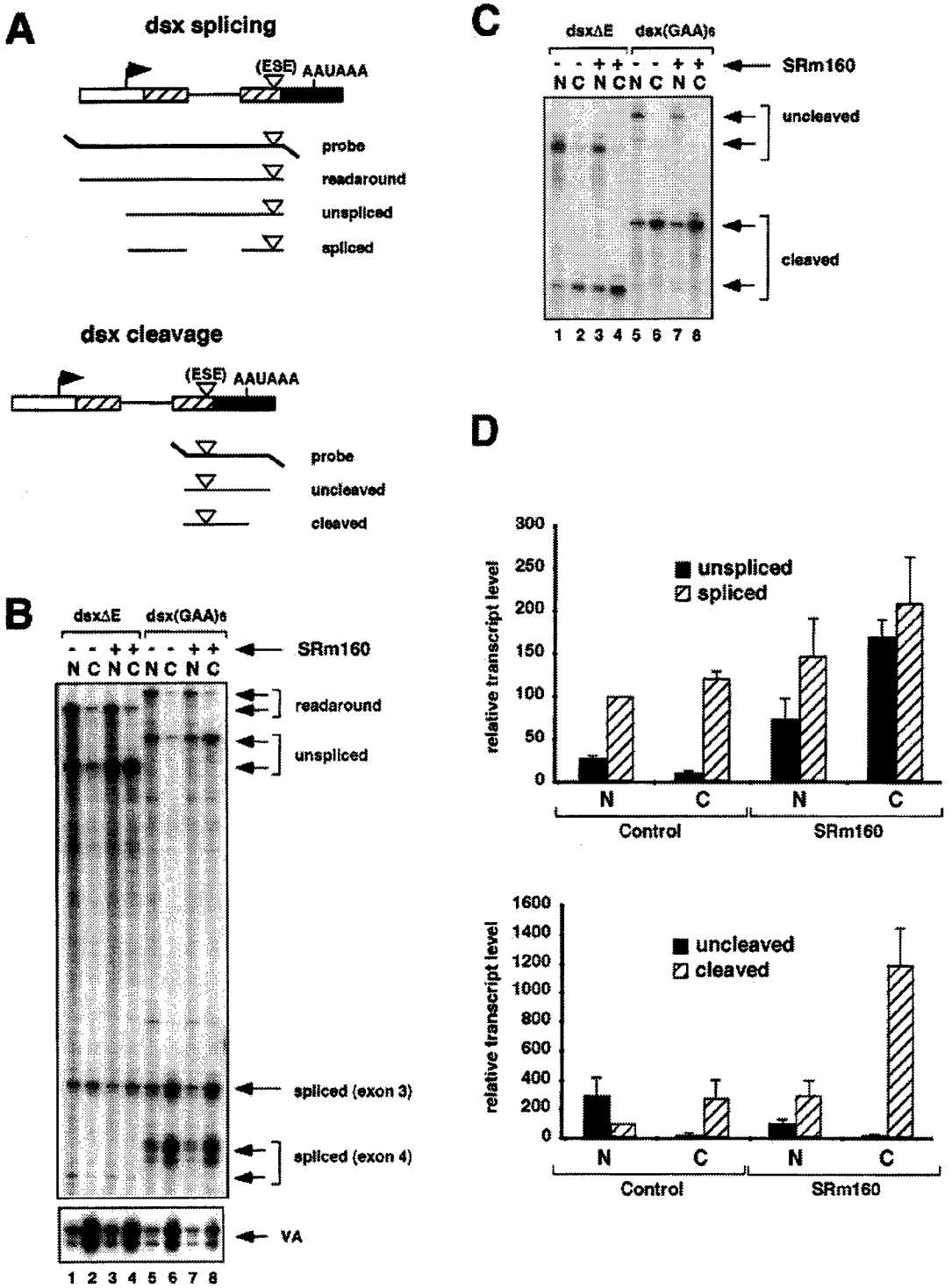

FIG. 2. Increased expression of SRm160 promotes 3′-end cleavage in vivo. (A) Schematic representation of the RNase protection probes used to analyze splicing and 3′-end cleavage in pre-mRNAs transcribed from reporters derived from exons 3 and 4 of the *Drosophila doublesex* gene (dsx). The dsx reporters contained either no ESE (dsxΔE) or a six-GAA repeat ESE [dsx$(GAA)_6$]. The predicted RNase protection products are shown below each probe (for sizes refer to Materials and Methods and supplementary information available at http://www.utoronto.ca/intron/supp_info). (B and C) Human 293 cells were transiently transfected with the dsxΔE (lanes 1 to 4) or dsx$(GAA)_6$ reporter (lanes 5 to 8) together with a control expression vector containing no insert (pcDNA3-Flag) (lanes 1 and 2 and 5 and 6) or an expression vector for Flag epitope-tagged SRm160 (pcDNA3-fSRm160) (lanes 3 and 4 and 7 and 8); the pol III reporter (pSPVA) was cotransfected in each case as an internal control.

fected with plasmids containing different pre-mRNA reporters, with or without an expression plasmid encoding SRm160 fused to an N-terminal FLAG epitope (pcDNA3-fSRm160). Each pre-mRNA reporter contained an upstream promoter derived from the adenovirus major-late region and a 3′-late poly(A) signal from simian virus 40 (SV40). Included in all transfections was an RNA pol III-VA RNA reporter plasmid (pSPVA), which serves as an internal control for monitoring the relative transfection efficiency and recovery of RNA between samples. RNA was isolated from both the nuclear and cytoplasmic fractions of the transfected cells and was analyzed by RNase protection in order to determine whether increased levels of SRm160 influence the nuclear-cytoplasmic distribution of transcripts, as well their processing.

Elevated levels of SRm160 in vivo result in the cytoplasmic accumulation of unspliced pre-mRNA transcripts. The effect of increased expression of SRm160 was first tested on the processing of a model substrate derived from exons 1 and 2 of human β-globin (hβ-glo) pre-mRNA (Fig. 1A). Immunoblotting experiments indicated that, in a typical transfection experiment, the level of fSRm160 was approximately 10- to 20-fold higher than the level of endogenous SRm160 (data not shown). RNA in the nuclear and cytoplasmic fractions was analyzed by using the RNase protection probe illustrated in Fig. 1A, which spans from 99 bases upstream of the start site of transcription to the 3′ end of exon 2. In the control transfection the hβ-glo transcripts were processed and exported efficiently, resulting in the accumulation of spliced mRNA almost exclusively in the cytoplasmic fraction (Fig. 1B, lanes 1 and 2). Surprisingly, however, when fSRm160 was expressed a high level of unspliced transcripts accumulated in the cytoplasmic fraction in addition to spliced mRNA (lanes 3 and 4). The size of the protected product indicates that these unspliced transcripts are correctly initiated. In several independent experiments the level of the VA RNA did not change significantly following fSRm160 expression (the increase observed between lanes 2 and 4 in Fig. 1B is less than twofold and most likely is due to variation in transfection efficiency; refer also to data in Fig. 2). Therefore, the effect of increased fSRm160 expression on promoting the cytoplasmic accumulation of unspliced hβ-glo transcripts appears to be specific for pol II transcripts. Thus, increased SRm160 expression appears to prevent or bypass splicing in addition to uncoupling interactions that normally would retain unspliced transcripts in the nucleus.

We next determined whether expression of fSRm160 influences the processing and nuclear-cytoplasmic distribution of another model transcript, derived from exons 3 and 4 of the *doublesex* gene of *Drosophila* (dsx pre-mRNA) (Fig. 2). The dsx pre-mRNA contains a suboptimal 3′-splice site–polypyrimidine tract and requires an exonic splicing enhancer (ESE) in exon 4 for efficient splicing. Previously it was shown that SRm160 is required for a mammalian ESE, consisting of 6xGAA repeats, to promote the splicing of this substrate in vitro (12). In order to investigate whether SRm160 influences the processing and/or nucleocytoplasmic distribution of this substrate in vivo, we next compared the effect of its increased expression on dsx reporters containing or lacking a 6xGAA repeat ESE in exon 4, designated dsx$(GAA)_6$ and dsxΔE, respectively (Fig. 2A).

RNA isolated from the nuclear and cytoplasmic fractions of cells transfected with the dsxΔE and dsx$(GAA)_6$ reporters, with or without pcDNA3-fSRm160, was analyzed by RNase protection using the probes illustrated in Fig. 2A. The dsx splicing probe spans from −99 bases upstream of the start site of transcription to the 3′ end of exon 4, allowing the detection of unspliced and spliced transcripts as well as readaround transcripts, which can arise by inefficient termination of transcription or incorrect initiation at cryptic promoters. In the absence of fSRm160 expression, insertion of the 6xGAA ESE in exon 4 resulted in an increase in the efficiency of splicing, similar to its activity observed in vitro (for an example, see reference 12) (Fig. 2B, compare lanes 1 and 2 with lanes 5 and 6). Consistent with numerous previous reports demonstrating the nuclear retention of unprocessed transcripts, the unspliced pre-mRNA and readaround transcripts from both reporter plasmids were detected primarily in the nuclear fractions (compare lanes 1 and 5 with lanes 2 and 6), whereas the majority of spliced transcripts from each reporter were detected in the cytoplasmic fractions (compare lanes 2, 4, 6, and 8 with lanes 1, 3, 5, and 7).

Similar to the results obtained with the hβ-glo reporter transcripts shown in Fig. 1, fSRm160 expression resulted in a pronounced increase in the level of unspliced pre-mRNA in the cytoplasmic fraction for both the dsxΔE and dsx$(GAA)_6$ transcripts (compare lanes 2 and 4 and lanes 6 and 8). The ratio of cytoplasmic to nuclear unspliced dsxΔE and dsx$(GAA)_6$ pre-mRNA transcripts increased eightfold and sevenfold, respectively (average values from four independent experiments), indicating that the effect is not dependent on the presence of the ESE (see Fig. 2D; data not shown). As observed for the hβ-glo reporter transcripts, the increase in cytoplasmic unspliced pre-mRNA did not coincide with a significant decrease in the level of pre-mRNA in the nuclear fractions, again indicating that fSRm160 expression results predominantly in the cytoplasmic accumulation of a population of unspliced transcripts. Also similar to the results with the hβ-glo transcripts, increased expression of fSRm160 did not significantly affect the levels of the spliced dsxΔE or dsx$(GAA)_6$ transcripts in the nuclear or cytoplasmic fractions (Fig. 2B, compare lanes 3 and 4 with 1 and 2 and lanes 7 and 8 with 5 and 6). In both cases

Proportional amounts of RNA isolated from the nuclear (N) and cytoplasmic (C) fractions of the transfected cells were analyzed by RNase protection using either the splicing protection probe (B) or the 3′-end protection probe (C). The identity of each RNA species is indicated. Note that exon 4 of the dsxΔE pre-mRNA, migrating near the bottom of the gel in panel B, is less strongly detected by the [^{32}P]UTP-labeled RNase protection probe due to the low A content of this exon. It was, however, readily detected following a longer exposure of the gel (data not shown). (D) Quantification of the effect of SRm160 expression on the yields of different RNA species transcribed from the dsxΔE reporter. RNA isolated from transfected cells was analyzed by RNase protection as described above, and the amounts of unspliced, spliced, uncleaved, and cleaved RNAs from three independent experiments were quantified. The values were normalized for both VA signal and U content. For the purpose of averaging different experiment values, the nuclear spliced RNA and the nuclear cleaved RNA were normalized to a value of 100.

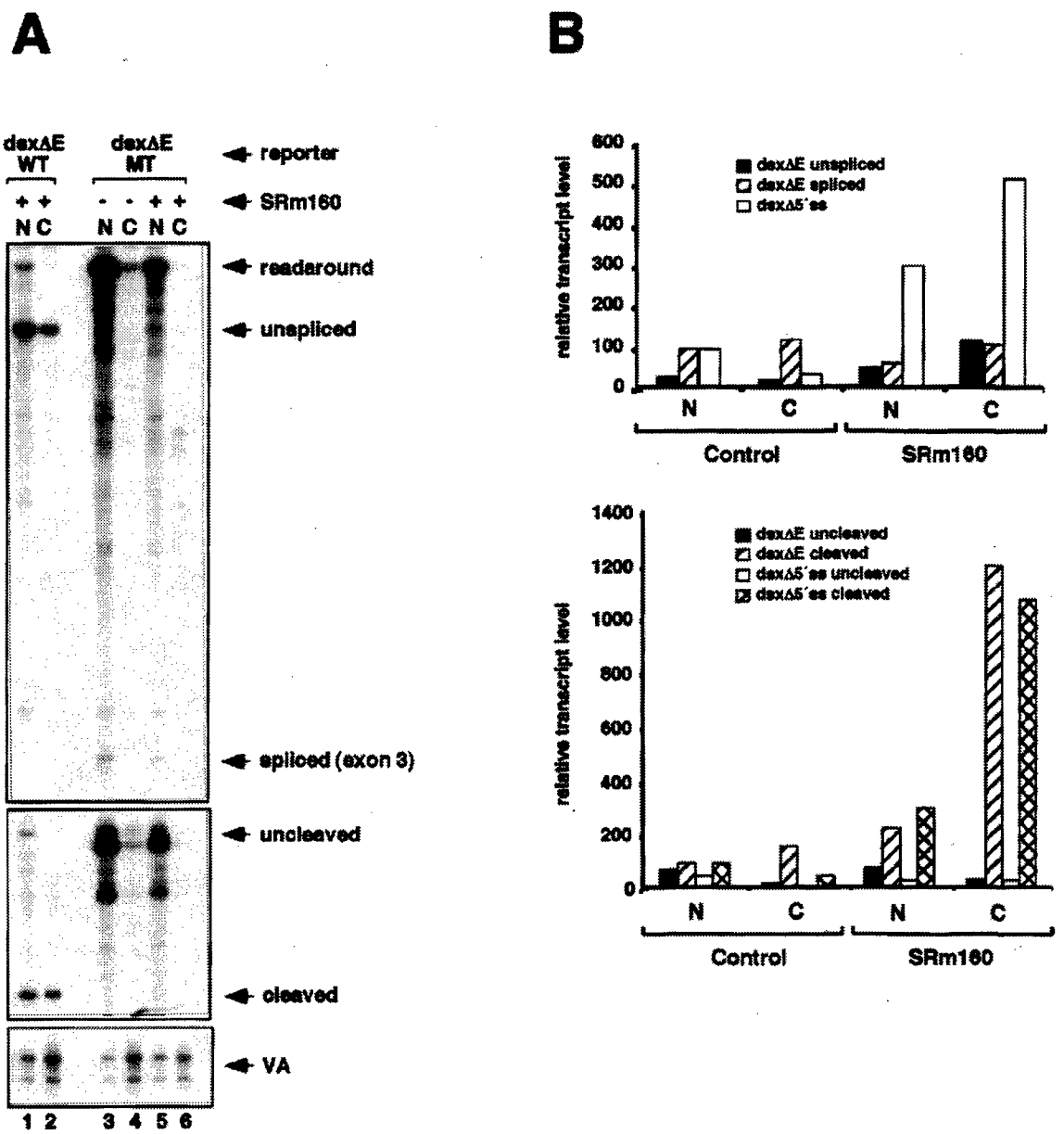

FIG. 3. Mutation of the polyadenylation signal, but not deletion of the 5′ splice site, prevents cleavage and cytoplasmic accumulation of unspliced RNA by SRm160. (A) 293 cells were transiently transfected with the dsxΔE reporter containing either a wild-type (dsxΔE-WT) (lanes 1 and 2) or mutant (dsxΔE-MT) (lanes 3 to 6) polyadenylation signal (see the text). The cells were cotransfected with a control, empty expression vector (pcDNA3-Flag) (lanes 3 and 4) or an expression vector for Flag epitope-tagged SRm160 (pcDNA3-fSRm160) (lanes 1 and 2 and 5 and 6) and with pSPVA as an internal control. Proportional amounts of RNA isolated from the nuclear (N) and cytoplasmic (C) fractions were analyzed by RNase protection using the splicing and cleavage probes shown in Fig. 2A. (B) Quantification of the effect of SRm160 expression on the nuclear and cytoplasmic levels of RNAs transcribed from a splicing-inactive dsx reporter, which lacks a functional 5′ splice site (dsxΔ5′ss), and the dsxΔE reporter. RNA isolated from transfected cells was analyzed by RNase protection as described in the legend to Fig. 2A, and the amounts of the unspliced, spliced, uncleaved, and cleaved RNAs were quantified as described in the legend to Fig. 2 (refer to supplementary information at the web site cited above for details of the dsxΔ5′ss reporter and protection probe).

it is possible that a population of pre-mRNA transcribed from these reporters is processed and exported prior to fSRm160 reaching levels which result in the cytoplasmic accumulation of unspliced transcripts (see Discussion). Thus, the results demonstrate that elevated levels of SRm160 can promote the cytoplasmic accumulation of distinct pre-mRNA transcripts.

SRm160 promotes the 3′-end cleavage of transcripts in vivo. A prerequisite for the nuclear export of unspliced pre-mRNA

is that it is released from nuclear retention factors, which can include transcribing RNA pol II as well as factors involved in the formation of splicing complexes (8, 10, 24, 42). Release of transcripts from pol II involves recognition of the AAUAAA poly(A) site by 3′-end cleavage factors, which is important for efficient transcription termination as well as 3′-end cleavage (2, 37). One possibility is that increased SRm160 expression allows the release of unspliced transcripts from the nucleus by facilitating 3′-end formation without the requirement for concomitant splicing. To investigate this we asked whether fSRm160 expression influences the 3′-end cleavage of the dsx transcripts. Accordingly, the same samples shown in Fig. 2B were analyzed with RNase protection probes designed to monitor 3′-end cleavage (Fig. 2C; refer to Fig. 2A).

Consistent with an important role for splicing in the promotion of 3′-end processing, in the absence of fSRm160 expression a significant increase in the ratio of cleaved to uncleaved transcripts was detected as a result of insertion of the 6xGAA ESE in exon 4 (Fig. 2C, compare lanes 1 and 2 with lanes 5 and 6). This increase was 9-fold in the nuclear fraction and 14-fold in the cytoplasmic fraction (data not shown). Moreover, in agreement with an important role for 3′-end processing in facilitating the release of transcripts from the nucleus, essentially all of the transcripts detected in the cytoplasmic fractions were 3′-end cleaved, whereas the uncleaved transcripts were almost entirely detected in the nuclear fractions (Fig. 2C, compare lanes 1 and 2 and lanes 5 and 6). Significantly, expression of fSRm160 resulted in a further increase [fourfold for dsxΔE and twofold for dsx$(GAA)_6$, as determined from averaging values from three independent experiments] in the level of cleaved transcripts in the cytoplasmic fractions, concurring with the increased levels of cytoplasmic unspliced transcripts in these fractions (compare lanes 4 and 8 with 2 and 6 in Fig. 2B and C; see also Fig. 2D) (data not shown). The consistently higher level of cleavage-stimulatory activity of fSRm160 observed for the dsxΔE transcripts compared to that of the dsx $(GAA)_6$ transcripts suggests that the level of cleavage promoted by the 6xGAA repeat ESE may already be near saturation. In agreement with the results obtained with the dsx pre-mRNA reporters and with the results in Fig. 1B, elevated levels of SRm160 also resulted in the presence of cleaved, unspliced, hβ-glo pre-mRNA transcripts in the cytoplasmic fractions (data not shown). Thus, elevated levels of SRm160 in vivo appear to facilitate the nuclear release of different unspliced transcripts by stimulating 3′-end cleavage.

Promotion of transcript release from the nucleus by SRm160 requires a wild-type polyadenylation signal. In order to confirm whether SRm160 acts to promote the nuclear release of unspliced transcripts by activating 3′-end formation, we compared its activity on dsxΔE transcripts containing either a wild-type (AAUAAA, dsxΔE-WT) or a mutant, inactive (AAGAAA, dsxΔE-MT), SV40 late poly(A) signal (Fig. 3A). In agreement with previous experiments indicating that the AAUAAA signal is required for 3′-end cleavage and for RNA pol II to terminate (2, 37), mutation of the poly(A) signal resulted in the accumulation to high levels in the nuclear fraction of uncleaved, readaround transcripts (Fig. 3A, lanes 3 and 5). Although these readaround transcripts probably arise as a consequence of the loss of efficient termination of transcription, we cannot exclude the alternative possibility that they also arise through incorrect initiation of transcription. However, in either case the results demonstrate that fSRm160 expression did not result in the 3′-end cleavage or cytoplasmic accumulation of the dsxΔE-MT transcripts. It is noteworthy that coexpression of fSRm160 did not result in a significant change in the level of readaround transcripts in the nuclear fraction; the slight decrease observed in Fig. 3A is probably due to experimental variation, since it was not observed in repeat experiments (lanes 3 and 5) (data not shown). This indicates that the cytoplasmic accumulation of unspliced transcripts following fSRm160 expression is not a consequence of increased levels of transcription from the reporter plasmids. Results similar to those described above were observed between wild-type and mutant poly(A) signal derivatives of the dsx$(GAA)_6$ and hβ-glo reporter transcripts (data not shown). These data confirm that activation of 3′-end cleavage by fSRm160 requires the presence of the AAUAAA poly(A) signal and also demonstrate that increased levels of SRm160 do not promote the nuclear release of transcripts without 3′-end cleavage.

SRm160 can promote the 3′-end cleavage and nuclear release of transcripts independently of splicing. The results so far indicate that elevated levels of SRm160 in vivo can promote the 3′-end cleavage and cytoplasmic accumulation of transcripts without the requirement for concomitant splicing. To confirm whether this is the case, we next compared the ability of SRm160 to promote these activities on a splicing-inactive derivative of the dsxΔE reporter, which contains a deletion in the 5′ splice site (dsxΔ5′ss) (Fig. 3B). RNase protection analysis with a probe spanning the intron and exon sequences of this transcript confirmed that it was not spliced (data not shown). Similar to the results observed for the unspliced dsxΔE transcripts, RNase protection analysis using the dsxΔE 3′-end cleavage probe (refer to Fig. 2A) revealed that elevated levels of SRm160 resulted in a pronounced increase in the level of cleavage and cytoplasmic accumulation of the dsxΔ5′ss transcript (Fig. 3B and data not shown). This confirms that the presence of a functional intron and splicing is not required in order for SRm160 to promote 3′-end cleavage and nuclear release of transcripts. As will be expanded on below, although excess levels of SRm160 can result in the activation of 3′-end cleavage independently of splicing it may normally only promote 3′-end cleavage when coupled to splicing.

Specificity of the cleavage-stimulatory and export activities of SRm160. In order to investigate the specificity of SRm160 in promoting 3′-end cleavage and cytoplasmic accumulation of transcripts in vivo, its activity was initially compared alongside the SRm160-interacting factor DEK, which (like SRm160) associates with splicing complexes and forms a component of a splicing-dependent exon junction complex (see the introduction). Transient transfection and RNase protection assays were performed as described above using the dsx$(GAA)_6$ pre-mRNA reporter together with an expression plasmid encoding DEK (Fig. 4A). Although DEK was efficiently expressed, unlike SRm160 it did not result in a significant level of stimulation of 3′-end cleavage or accumulation of pre-mRNA in the cytoplasm (Fig. 4A, compare lanes 3 and 4 with lanes 1 and 2 and lanes 5 and 6) (data not shown).

Several FLAG epitope-tagged deletion derivatives of SRm160 were also tested for their ability to stimulate cleavage and cytoplasmic accumulation of transcripts. Although these de-

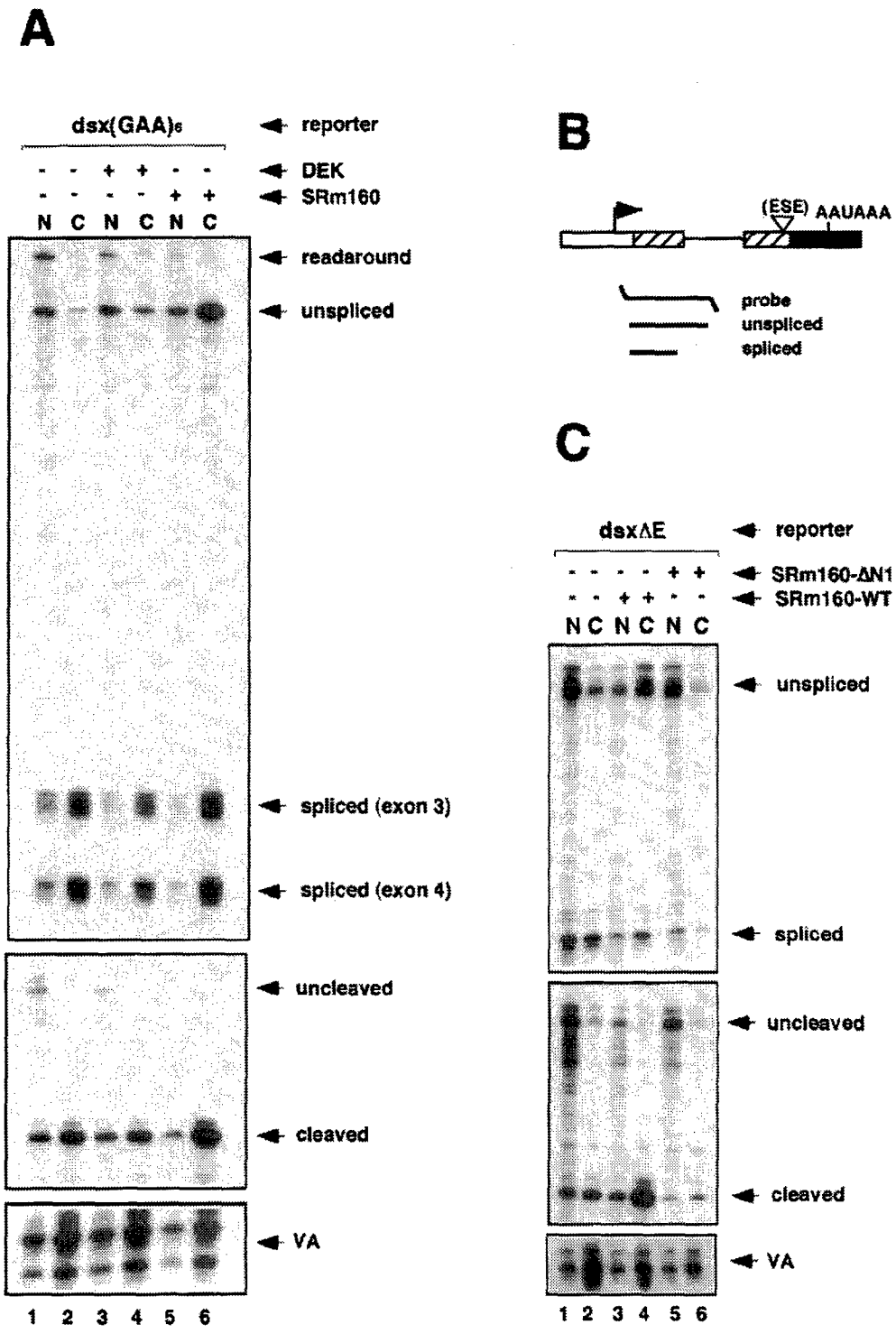

FIG. 4. Specificity of the cleavage-stimulatory and transcript export activities of SRm160. (A) Human 293 cells were transiently transfected with the dsx(GAA)$_6$ reporter together with a control expression vector containing no insert (pcDNA3-Flag) (lanes 1 and 2), an expression vector for HA epitope-tagged DEK (pcDNA3-DEK) (lanes 3 and 4), or an expression vector for Flag epitope-tagged SRm160 (pcDNA3-fSRm160) (lanes 5 and 6); the pol III reporter (pSPVA) was cotransfected in each case as an internal control. Proportional amounts of RNA isolated from the nuclear (N) and cytoplasmic (C) fractions of the transfected cells were analyzed by RNase protection using either a splicing protection probe or the 3′-end protection probe (refer to the legend to Fig. 2). The identity of each RNA species is indicated. (B) Schematic representation of the short

letion proteins were expressed as efficiently as wild-type fSRm160, the majority did not promote 3′-end cleavage or cytoplasmic–pre-mRNA accumulation of dsx reporter transcripts (data not shown). However, interpretation of the results was complicated, since many of the inactive deletion derivatives did not localize in the same manner as wild-type SRm160, raising the possibility that their lack of function could be a consequence of mislocalization. However, one of the deletions (fSRm160ΔN1), which lacks residues 1 to 155 corresponding to the highly conserved N-terminal domain of SRm160, localized in the same nuclear speckled pattern as wild-type SRm160 yet was not active in promoting the 3′-end cleavage and cytoplasmic accumulation of transcripts. This is demonstrated in the RNase protection analysis of dsxΔE pre-mRNA splicing and cleavage shown in Fig. 4C (compare lanes 3 and 4 with lanes 1 and 2 and lanes 5 and 6), in which cleavage was analyzed with the same probe as that described for Fig. 2A and splicing was analyzed by using the probe illustrated in Fig. 4B. This latter probe spans from the start of exon 3 to the middle of the dsx intron. It is noteworthy that, while inactive, SRm160ΔN1 retains the arginine/serine (RS) domain and other repeat motifs that are probably important for its correct localization and interactions with other pre-mRNA processing factors. This result indicates that intact SRm160 is important for stimulation of 3′-end cleavage and cytoplasmic accumulation of pre-mRNA and that both of these activities depend on the presence of the conserved N-terminal domain of the protein.

SRm160 interacts with the CPSF. The activity of SRm160 in promoting 3′-end processing in the experiments described above could result from one or more indirect effects arising from its increased expression levels or the more interesting possibility that it participates more directly in promoting 3′-end cleavage. In order to distinguish between these possibilities we next asked whether SRm160 associates with one or more components of the 3′-end cleavage machinery.

Immunoprecipitates were collected from HeLa nuclear extract by using a MAb specific for SRm160 (MAb-B1C8) (6, 45). MAb-B1C8 has previously been shown to immunoprecipitate SRm160 in complexes containing SR family proteins, the SR-related proteins hTra2-beta and Hel117, and the oncoprotein DEK (12, 31) (our unpublished observations). The MAb-B1C8 immunoprecipitates were immunoblotted with available antisera specific for 3′-end processing factors, including CstF-77 (cleavage stimulation factor 77-kDa subunit), CPSF-160 (cleavage polyadenylation specificity factor 160-kDa subunit), and PAP [poly(A) polymerase]. Although all three of these antibodies detected proteins of the expected sizes in HeLa nuclear extract, only CPSF-160 was significantly enriched in the MAb-B1C8 immunoprecipitates (Fig. 5A, lane 4, and data not shown). Approximately 2% of CPSF-160 in the nuclear extract was immunoprecipitated with MAb-B1C8, indicating that only a low level of this cleavage factor interacts with SRm160 or else that this interaction is unstable during immunoprecipitation. Nevertheless, the interaction was specific, since CPSF-160 was not substantially coimmunoprecipitated with excess amounts of nonspecific control antibodies (lanes 3 and 5 and data not shown). Moreover, CPSF-160 was still coimmunoprecipitated by MAb-B1C8 after extensive pretreatment of the nuclear extract with RNase (Fig. 5B, compare lanes 1 and 2), indicating that it probably associates with SRm160 through protein-protein interactions (Fig. 5A, compare lanes 4 and 6).

In order to confirm whether SRm160 and CPSF associate specifically, a reciprocal immunoprecipitation experiment was performed in which immunoprecipitates were collected from RNase-pretreated HeLa nuclear extract using antisera specific for the 73-kDa subunit of CPSF (CPSF-73), PAP, and CstF-77 and then were immunoblotted with MAb-B1C8 (Fig. 5C). All of these antibodies immunoprecipitate their target proteins efficiently, whereas the anti-CPSF-160 antibody used above does not and therefore was not included in the analysis (data not shown). The anti-CPSF-73 antibody resulted in a significant level of enrichment of SRm160 (lane 4), whereas little or no coimmunoprecipitation was observed above the background level with a control antibody, the anti-PAP antibody, or the anti-CstF-77 antibody (compare lane 3 with lanes 5 and 6, respectively). Approximately 2 to 3% of SRm160 was coimmunoprecipitated with anti-CPSF-73, again indicating that a relatively low level of SRm160 and CPSF associate specifically in HeLa nuclear extract.

SRm160 stimulates 3′-end cleavage in vitro. The activity of SRm160 in promoting 3′-end processing was next investigated by determining whether it can activate 3′-end cleavage of different RNA substrates with functional polyadenylation sites in vitro (Fig. 6). To first determine whether SRm160 can promote cleavage independent of splicing, highly purified baculovirus-expressed SRm160 (bSRm160) (Fig. 6A) (see reference 3) was added to 3′-end cleavage reaction mixtures containing a substrate derived from the 3′-half of exon 4 of the dsxΔE pre-mRNA, fused to either a wild-type [AAUAAA, dsxΔ-p(A)-WT] or mutant [AAGAAA, dsxΔ-p(A)-MT] late poly(A) signal from SV40 (Fig. 6B). Significantly, increasing amounts of bSRm160 stimulated, up to approximately threefold, cleavage of the dsxΔ-p(A)-WT but not the dsxΔ-p(A)-MT substrate (Fig. 6B, compare lanes 1 to 3 with lanes 4 to 6; note that although these substrates differ only by the U→G substitution in the AAUAAA sequence, they migrate differently due to a structural difference conferred by this substitution). The results demonstrate that SRm160, similar to its activity in vivo, can promote 3′-end cleavage in vitro. Moreover, its cleavage-

splicing RNase protection probe used to analyze splicing of transcripts from the dsxΔE reporter. The predicted RNase protection products are shown below each probe (for sizes refer to Materials and Methods and supplementary information available at http://www.utoronto.ca/intron/supp_info). (C) Human 293 cells were transiently transfected with the dsxΔE reporter together with a control expression vector containing no insert (pcDNA3-Flag) (lanes 1 and 2), an expression vector for Flag epitope-tagged SRm160 (pcDNA3-fSRm160) (lanes 3 and 4), or an expression vector for Flag epitope-tagged SRm160 deleted from amino acids 1 to 155 (pcDNA3-fSRm160ΔN1) (lanes 5 and 6); the pol III reporter (pSPVA) was cotransfected in each case as an internal control. Proportional amounts of RNA isolated from the nuclear (N) and cytoplasmic (C) fractions of the transfected cells were analyzed by RNase protection using either the short splicing protection probe or the 3′-end protection probe. The identity of each RNA species is indicated.

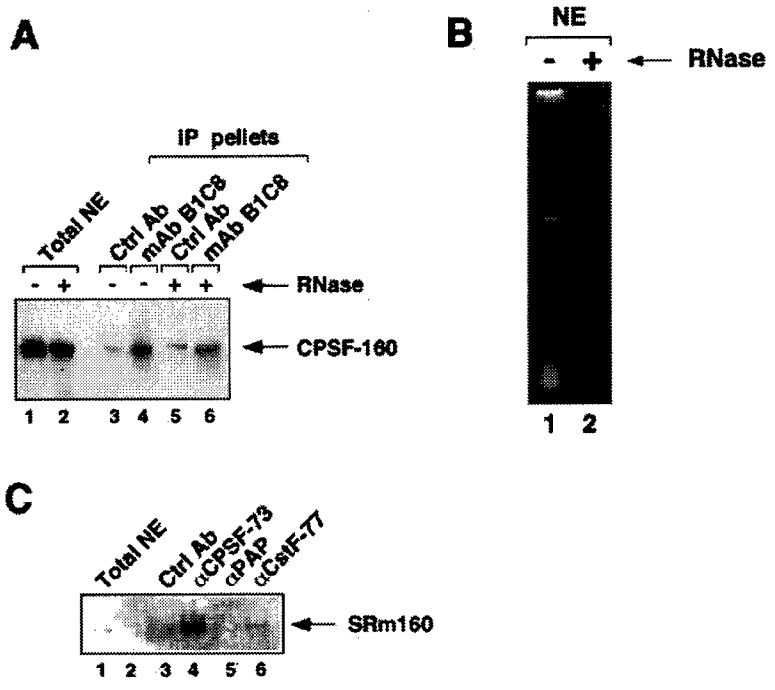

FIG. 5. SRm160 associates with the 3′-end cleavage machinery. (A) Immunoprecipitates (IP) were collected from HeLa nuclear extract (NE) by using the SRm160-specific MAb (MAb-B1C8) (lanes 4 and 6) and excess levels of a control antibody (rabbit anti-mouse immunoglobulin; Ctrl Ab) (lanes 3 and 5). The immunoprecipitates were separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel and immunoblotted with an affinity-purified antibody specific for the 160-kDa subunit of the human CPSF (CPSF-160). Total nuclear extract, separated in lanes 1 and 2, represents 7% of the amount of extract used in each immunoprecipitation. Nuclear extract was preincubated in the presence (lanes 2, 5, and 6) or absence (lanes 1, 3, and 4) of RNase prior to immunoprecipitation. (B) Analysis of the RNA content of the nuclear extract used for immunoprecipitation shown in panel A. RNA isolated from nuclear extract pretreated with (lane 2) or without (lane 1) RNase was analyzed on a 10% denaturing acrylamide gel stained with ethidium bromide. (C) Immunoprecipitates were collected from RNase-pretreated HeLa nuclear extract by using rabbit polyclonal antibodies to the 73-kDa subunit of human CPSF (CPSF-73) (lane 4), PAP (lane 5), CstF-77 (lane 6), and a control antibody (rabbit anti-glutathione *S*-transferase) (lane 3). The immunoprecipitates were separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel and immunoblotted with MAb-B1C8. Total nuclear extract, separated in lane 1, represents ~0.5% of the amount of extract used in each immunoprecipitation.

stimulatory activity does not depend on the presence of active splice sites.

Specificity of the cleavage-stimulatory activity of SRm160 in vitro. In order to investigate the specificity of the 3′-end cleavage-stimulatory function of SRm160 in vitro, we compared its activity with two different baculovirus-expressed SR proteins, SRp30c and SRp40 (Fig. 6C). Both of these proteins were purified to near homogeneity and were active in splicing reconstitution assays performed in HeLa S100 reactions (data not shown). Titration of equal amounts of these proteins as SRm160 in 3′-cleavage reaction mixtures containing the dsxΔ-p(A)-WT substrate did not significantly influence the ratio of cleaved to uncleaved substrate, whereas an approximately fourfold increase was consistently observed for SRm160 (Fig. 6C and data not shown). These differences in cleavage-stimulatory activity are not a consequence of differences in the lengths of the RS domains of these proteins, since SRp40 contains a higher number of consecutive SR/RS repeats than SRm160 whereas SRp30c contains fewer repeats. Moreover, we have observed that multiple domains of SRm160 other than the RS-rich regions of the protein are important for promoting 3′-end cleavage in vivo (see Fig. 4C) (our unpublished observations). Thus, the results indicate that the activity of SRm160 in stimulating 3′-end processing in vitro is not a general feature of RS domain proteins and, moreover, does not reflect the length of the RS domains of these splicing factors.

SRm160 augments the splicing-dependent enhancement of 3′-end cleavage in vitro. The experiments presented so far demonstrate a cleavage-stimulatory activity of SRm160 that can function independently of splicing. However, at endogenous levels SRm160 normally associates stably only with transcripts in the context of functional splicing complexes (5, 12) and may, therefore, provide an important role in 3′-end formation coupled to splicing. To investigate whether the 3′-end cleavage-stimulatory property of SRm160 is augmented by the formation of functional splicing complexes, we next compared its influence on the 3′-end cleavage of splicing-active and -inactive derivatives of an adenovirus-derived pre-mRNA substrate (MXSVL) (35), each containing the late poly(A) signal from SV40 (Fig. 6D and E). Importantly, bSRm160 was added to splicing and cleavage reaction mixtures containing these substrates at levels that were stimulatory to splicing, thus allowing its activity in 3′-end cleavage to be assessed in the context of productive splicing complex formation.

Splicing and cleavage reaction mixtures containing wild-type MXSVL (Fig. 6D) or a splicing-inactive derivative lacking a 5′

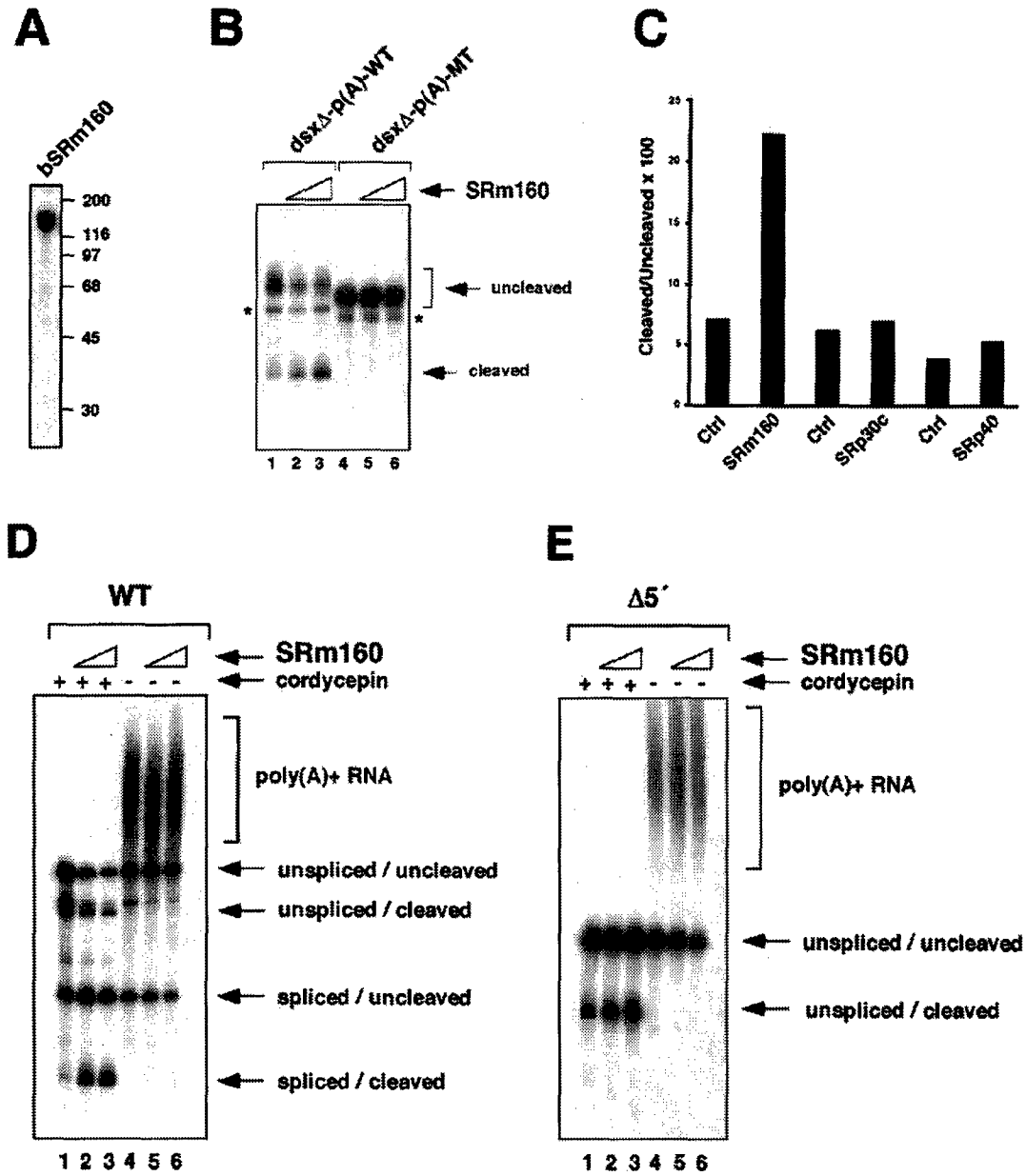

FIG. 6. Highly purified, recombinant SRm160 preferentially stimulates cleavage of spliced substrates in vitro. (A) Analysis of recombinant, baculovirus-expressed SRm160 (bSRm160) by sodium dodecyl sulfate gel electrophoresis and Coomassie blue staining (see Materials and Methods for purification details). bSRm160 (4.5 μg) was loaded on the gel shown. (B) bSRm160 stimulates the 3′-end cleavage of a dsx substrate lacking functional splice sites in vitro. Reactions were performed with substrates derived from the 3′ half of dsx exon 4 (no ESE present) containing either a wild-type SV40 late poly(A) site [dsxΔ-p(A)-WT] (lanes 1 to 3) or a mutant poly(A) site [dsxΔ-p(A)-MT] (lanes 4 to 6) in the presence (lanes 2 and 3 and 5 and 6) or absence (lanes 1 and 4) of bSRm160. bSRm160 (165 ng) was added to the reaction shown in lanes 2 and 5, and 330 ng of bSRm160 was added to the reaction shown in lanes 3 and 6. The reactions were performed with added 5′-cordycepin triphosphate, as described in Materials and Methods. Duplicate reactions performed in the absence of 5′-cordycepin triphosphate confirmed the identity of the 3′-end cleaved bands (data not shown). Asterisks indicate a nonspecific degradation product not related to cleavage. (C) Specificity of the 3′-end stimulatory activity of SRm160. Approximately equal amounts (176 ng) of bSRm160, bSRp30c, and bSRp40, as assessed by Bradford assay, were added to 3′-end cleavage reaction mixtures incubated with the dsxΔ-p(A)-WT substrate. The reaction products were analyzed by electrophoresis on a denaturing gel and quantified by using a Molecular Dynamics PhosphorImager and ImageQuant software. (D and E) In vitro splicing and cleavage reactions containing a wild-type (WT) (D) or a 5′-splice site-deleted (Δ5′) (E) adenovirus pre-mRNA substrate, with an SV40 late polyadenylation signal (MSXVL) (35), were performed in the presence (lanes 2 and 3 and 5 and 6) or absence (lanes 1 and 4) of bSRm160. bSRm160 (110 ng) was added to the reaction shown in lanes 2 and 5, and 220 ng of bSRm160 was added to the reaction shown in lanes 3 and 6. The reactions were performed with (lanes 1 to 3) or without (lanes 4 to 6) added 5′-cordycepin triphosphate in order to distinguish the 3′-end cleaved products (see the text).

splice site MXSVL (Δ5′) (Fig. 6E) were incubated with or without the ATP analog 5′-cordycepin triphosphate in order to distinguish the 3′-end cleaved products. In the presence of 5′-cordycepin triphosphate, polyadenylation is prevented, resulting in the accumulation of 3′-end cleaved products. In the absence of 5′-cordycepin triphosphate, the 3′-end cleaved products are polyadenylated and migrate as a smear in the upper region of the gel (Fig. 6D and E, compare lanes 1 to 3 with lanes 4 to 6). The different products detected in the reactions with the MXSVL substrates correspond to those previously characterized in detail (34, 35) and were assigned accordingly.

Quantification of the reaction intermediates and products revealed that, at the highest level of bSRm160 addition (220 ng) to reaction mixtures containing the WT-MXSVL substrate, only minor (less than twofold) changes in the level of unspliced and cleaved pre-mRNA and spliced and uncleaved transcripts were observed. However, there was a sevenfold increase in the level of spliced and cleaved mRNA (Fig. 6D, compare lanes 1 to 3) (data not shown). In contrast, addition of 220 ng of bSRm160 to reaction mixtures containing the Δ5′-MXSVL substrate resulted in an approximately threefold increase in the level of cleavage of this pre-mRNA (Fig. 6E, compare lanes 1 to 3). Similarly, addition of 220 ng of bSRm160 to an MXSVL substrate lacking a functional 3′ splice site also resulted in an approximately threefold increase in 3′-end cleavage (data not shown). Thus, in agreement with the results obtained with the dsxΔ-p(A) substrate (Fig. 6B), SRm160 can promote 3′-end cleavage of the MXSVL substrate independently of splicing. However, the presence of functional splice sites and splicing of the MXSVL pre-mRNA appears to augment the activity of bSRm160 in promoting 3′-end cleavage. These results, taken together with the data shown in Fig. 5 indicating that SRm160 can associate with CPSF, provide evidence that SRm160 participates in the coupling of splicing and 3′-end processing.

DISCUSSION

The results of the present study provide new information on the coordination of splicing with 3′-end formation and the nuclear-cytoplasmic transport of transcripts. SRm160, which previously was shown to promote both constitutive and exon enhancer-dependent splicing, was found to stimulate 3′-end cleavage. At elevated levels in vivo, SRm160 increased the levels of distinct pre-mRNAs, consistent with previous evidence that its specific ratio to other splicing factors is critical for optimal splicing. Under these conditions, SRm160 activated the 3′-end cleavage of the unspliced transcripts, thereby circumventing the normal requirement for splicing to promote the cleavage of these substrates. A consequence of the activation of pre-mRNA 3′-end cleavage by SRm160 was the accumulation of the unspliced transcripts in the cytoplasm. Thus, elevated levels of SRm160 appear to bypass the normal requirement of splicing for the nuclear release of transcripts. Consistent with a more direct role in activating the 3′-end formation of transcripts, SRm160 was found to associate with CPSF and to promote 3′-end cleavage in vitro. Importantly, although SRm160 promoted 3′-end cleavage independently of splicing, its cleavage-stimulatory activity was enhanced by the concomitant splicing of a transcript in vitro. In summary, the results demonstrate a role for SRm160 in 3′-end cleavage and provide evidence that the level of this splicing coactivator is important not only for optimal splicing but also for the coordination of splicing with 3′-end formation and nuclear retention of incompletely processed transcripts.

Coupling of splicing and 3′-end formation. The mechanism(s) by which the splicing and 3′-end processing machineries communicate with each other is not well understood. Several reports have provided evidence for an important role for U1 snRNP components in 3′-end formation. Antibodies to Sm and U1 snRNP proteins were shown to inhibit polyadenylation in vitro (32). Subsequently, it was shown that U1 snRNA cross-links to polyadenylation efficiency elements upstream of the poly(A) site and that the efficiency of this cross-linking correlates with the efficiency of 3′-end formation (47). It was also reported that the U1 snRNP-A protein can interact with CPSF and promote increased polyadenylation in vitro (29). Other studies indicated that the binding of U1 snRNP and the SR family protein SRp20 to an intronic splicing enhancer sequence within the alternatively spliced, calcitonin/calcitonin gene-related peptide pre-mRNA correlates with increased 3′-end processing at an adjacent poly(A) site (27). In other contexts, both U1 snRNP-A and -70k proteins inhibit polyadenylation by interacting with poly(A) polymerase, whereas an interaction between poly(A) polymerase and the U2AF-65kDa subunit has been shown to increase splicing efficiency (16, 17, 43).

The association of SRm160 with pre-mRNA splicing substrates in vitro is normally strongly dependent on U1 snRNP and is further promoted by SR family proteins and U2 snRNP. It is possible that the activity of one or more U1 snRNP components promoting 3′-end formation described above could involve interactions mediated by SRm160. Although in the present study CPSF-160 was found to associate with SRm160, this interaction could be bridged by one or more intermediary factors. For example, in previous studies it was found that SRm160 interacts with several SR family and SR-related proteins (unpublished observations and references 5 and 12). Moreover, a recent report indicates that CPSF interacts indirectly with the cleavage factor (CF) I_m (11), the 68-kDa subunit of which, like SRm160, is an SR-related protein (41). Since the alternating RS domains of SR family and SR-related proteins interact and are important for the formation of protein-protein interactions, it is possible that the RS domain of CF I-68 could interact with one or more SR family and/or SR-related splicing factors, including SRm160.

A possible role for RS domain proteins (other than SRm160) in modulating 3′-end cleavage is supported indirectly by previous observations. A 22-nucleotide element from the histone *H2a* gene, which promotes both the 3′-end formation and export of transcripts, binds to the SR family proteins 9G8 and SRp20 (20). Although antibodies to these SR family proteins inhibited mRNA export, it was not determined whether they also interfered with cleavage. Elevated expression of the SR family protein SC35 and the SR-related, helicase-like protein HRH1(hPRP22), like that of SRm160, can result in the cytoplasmic accumulation of pre-mRNA, although the mechanism(s) underlying these effects was not investigated (36, 46). Our results suggest that these factors could facilitate the nuclear release of transcripts by stimulating cleavage, perhaps in

association with SRm160, or through functionally related yet distinct interactions. However, it is important to note that the effects we have observed for SRm160 are not general properties of RS domain proteins. Expression of elevated levels of the SR family protein ASF/SF2 does not result in 3′-end cleavage or cytoplasmic accumulation of transcripts (46; S. McCracken and B. J. Blencowe, unpublished observations), and elevated expression of U1-70K inhibits splicing but prevents the export of transcripts (40). Moreover, in the present study we have shown that, unlike SRm160, the SR family proteins SRp30c and SRp40 do not significantly influence 3′-end cleavage in vitro. Thus, SRm160 may be representative of a specific subset of SR proteins that can influence 3′-end processing and the nuclear-cytoplasmic distribution of transcripts.

SRm160 and the nuclear export of transcripts. In order for RNA pol II transcripts to be efficiently exported from the nucleus, they must first be released from nuclear retention factors. Recognition of splicing signals by factors that function early in the formation of splicing complexes is important for the retention of unspliced pre-mRNA in the nucleus (8, 10, 24, 42). Elevated expression levels of SRm160 resulted in the accumulation of unspliced transcripts in the cytoplasm, suggesting that it can either prevent, or else bypass, splicing complex formation. Moreover, the accumulation of unspliced transcripts in the cytoplasm suggests that excess SRm160 might result in the bypass of processes that normally degrade unprocessed RNA in the nucleus. In conjunction with these roles, it is also possible that excess SRm160 prematurely activates an mRNA export pathway, allowing efficient export of unprocessed transcripts (see below). However, regardless of the mechanism(s) by which increased SRm160 expression results in the accumulation of unspliced transcripts in the cytoplasm, the present results demonstrate that SRm160 can facilitate the release of transcripts from nuclear retention by stimulating 3′-end formation. In particular, increased levels of SRm160 did not relieve the nuclear retention of unspliced transcripts containing a mutant poly(A) site, indicating that its ability to promote the nuclear release of unspliced transcripts is, at least in part, a consequence of its ability to stimulate the 3′-cleavage of these transcripts.

It is important to note that, although excess levels of SRm160 could stimulate 3′-end formation independently of splicing, levels of SRm160 that were not inhibitory to splicing were more efficient in stimulating the 3′-end cleavage of splicing-active than of splicing-inactive pre-mRNAs in vitro. Taken together with previous results demonstrating a requirement for U1 snRNP and SR family proteins for the association of SRm160 with pre-mRNA (5, 12), these findings suggest that SRm160 normally promotes 3′-end formation during the formation of productive splicing complexes. Furthermore, recent work has shown that SRm160 forms part of a splicing-dependent complex, 20 to 24 nucleotides upstream of exon-exon junctions, that contains several factors, including DEK, Y14, RNPS1, and the mRNA export factor REF/Aly (25, 49). It is therefore possible that the association of SRm160 with this complex at 3′-most exon-exon junctions might facilitate mRNA export by forging interactions with one or more export factors, including REF, as well as by promoting 3′-end cleavage. Similarly, increased expression of SRm160 could result in the recruitment of export factors to unspliced transcripts, thereby facilitating their nuclear release at a step in addition to 3′-end formation.

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The Acute Myeloid Leukemia-associated Protein, DEK, Forms a Splicing-dependent Interaction with Exon-product Complexes

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Abstract. DEK is an ~45-kD phosphoprotein that is fused to the nucleoporin CAN as a result of a (6;9) chromosomal translocation in a subset of acute myeloid leukemias (AMLs). It has also been identified as an autoimmune antigen in juvenile rheumatoid arthritis and other rheumatic diseases. Despite the association of DEK with several human diseases, its function is not known. In this study, we demonstrate that DEK, together with SR proteins, associates with the SRm160 splicing coactivator in vitro. DEK is recruited to splicing factor-containing nuclear speckles upon concentration of SRm160 in these structures, indicating that DEK and SRm160 associate in vivo. We further demonstrate that DEK associates with splicing complexes through interactions mediated by SR proteins. Significantly, DEK remains bound to the exon-product RNA after splicing, and this association requires the prior formation of a spliceosome. Thus, DEK is a candidate factor for controlling postsplicing steps in gene expression that are influenced by the prior removal of an intron from pre-mRNA.

Key words: SR proteins • spliceosome • mRNP • mRNA • transport

Introduction

Increasing evidence suggests that different processes in gene expression communicate and are functionally coordinated with each other. For example, it has been demonstrated that transcription factors can influence the processing of a pre-mRNA. In this case, splicing and selection of polyadenylation sites can be influenced by promoter sequences that drive transcription by RNA polymerase II (pol II)[1] (Leroy et al., 1991; Cramer et al., 1997, 1999). Moreover, the COOH-terminal domain (CTD) of pol II is important for efficient capping, splicing, and 3′ end cleavage of nascent pre-mRNA (Cho et al., 1997; McCracken et al., 1997a,b). Splicing and 3′ end formation also influence each other, and interactions between these processes are important for the definition of terminal exons in pre-mRNA (Niwa and Berget, 1991; Wassarman and Steitz, 1993; Nesic and Maquat, 1994; Gunderson et al., 1997; Bauren et al., 1998; Lou et al., 1998; Vagner et al., 2000). Recently, it was demonstrated that the transport of mRNA from the nucleus to the cytoplasm is promoted by prior splicing, since mRNAs that had not been generated by splicing were inefficiently transported (Luo and Reed, 1999). Currently, it is not understood how individual steps in the gene expression pathway communicate with each other, although several lines of evidence indicate that proteins containing domains rich in alternating arginine and serine residues (RS domains), including several splicing factors, may be important for the coupling of splicing to other steps in pre-mRNA metabolism (reviewed in Fu, 1995; Corden and Patturajan, 1997; Blencowe et al., 1999).

Pre-mRNA splicing involves the step-wise assembly of large RNA-protein complexes, termed spliceosomes (reviewed in Kramer, 1996; Reed and Palandjian, 1997; Burge et al., 1999). The major spliceosome contains four small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4/U6, and U5) and many non-snRNP splicing factors. A large number of non-snRNP splicing factors con-

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[1]*Abbreviations used in this paper:* AML, acute myeloid leukemia; CTD, COOH-terminal domain; ESE, exonic splicing enhancer; EV, epidermodysplasia verruciformis; hnRNP, heteronucleoriboprotein; HPV, human papillomavirus; pets, peri-ets; rAb, rabbit polyclonal antibody; RNA pol II, RNA polymerase II; RS domain, domain rich in alternating arginine and serine residues; snRNPs, small nuclear ribonucleoprotein particles; SR protein, serine/arginine-repeat protein.

tain RS domains. Among these are members of the SR family, which share a common domain structure consisting of one or two NH_2-terminal RNA recognition motifs and a phosphorylated COOH-terminal RS domain (Fu, 1995; Manley and Tacke, 1996; Valcarcel and Green, 1996). Other RS domain-containing non-snRNP splicing factors have a distinct structural organization and are referred to as SR-related proteins (Fu, 1995; Blencowe et al., 1999). These include factors such as the alternative splicing regulators Tra, Tra2, and SWAP, as well as the subunits of the SRm160/300 splicing coactivator. The RS domains of SR family and SR-related proteins form interactions with each other and with snRNP-associated proteins that contain RS domains, and increasing evidence indicates that these interactions are critical for the formation of networks of interactions that promote splice site recognition across both intron and exon sequences.

Several SR-related proteins and interacting factors have been identified recently that are associated with transcription and 3′ end processing. These include the E2 protein of the epidermodysplasia verruciformis (EV)-associated human papillomavirus (HPV), which binds to promoter sequences and is important for the transcriptional regulation of viral genes. The EV-HPV E2 protein contains an RS hinge domain that interacts with serine/arginine-repeat proteins (SR proteins) involved in splicing, and appears to promote the splicing of pre-mRNAs transcribed from promoters activated specifically by the EV-HPV E2 protein (Lai et al., 1999). A class of SR-related proteins, referred to as SCAFs (SR-like CTD-associated factors), has been identified that preferentially bind to the hyperphosphorylated CTD of RNA pol II, which is the form of the CTD associated with elongating transcription complexes (Yuryev et al., 1996; Bourquin et al., 1997; Tanner et al., 1997; Patturajan et al., 1998). An SR-related protein (PCG-1) has been identified that functions as a transcriptional coactivator of nuclear receptors, which is implicated in adaptive thermogenesis in mice (Puigserver et al., 1998). Another new transcriptional coactivator (p52) has been identified that lacks an RS domain, but interacts with the SR family protein ASF/SF2, as well as with several transcription factors (Ge et al., 1998). The 68-kD subunit of the mammalian cleavage factor (CFIm), which functions in the 3′ end of processing transcripts, was recently isolated and found to contain an RS domain (Ruegsegger et al., 1998). These studies indicate that a variety of proteins containing RS domains are candidates for factors that coordinate different steps during the synthesis and processing of pre-mRNA.

SR family and SR-related proteins have been implicated in steps downstream of pre-mRNA splicing. Several SR proteins, including SRm160/300 and ASF/SF2, remain bound preferentially to the ligated exon-product, but not the intron-lariat product of the splicing reaction, which associates with snRNPs (Blencowe et al., 1998, 2000; Hanamura et al., 1998). A subset of SR family proteins shuttle between the nucleus and the cytoplasm, suggesting that they could function in the nuclear–cytoplasmic transport of mRNA (Cáceres et al., 1998). Recently, it was observed that complexes formed on exon-product RNAs generated by splicing are larger and more efficiently transported to the cytoplasm than complexes formed on the same RNAs that have not been through splicing (Luo and Reed, 1999). This observation suggests that there are factors that are targeted for association with mRNA by the splicing machinery, and may relate to numerous earlier observations that introns are important for the efficient expression of genes.

In the present study, we have identified the oncoprotein DEK as a splicing complex component that remains associated with spliced exons dependent on prior splicing of pre-mRNA. Furthermore, we show that the association of DEK with splicing complexes is mediated by specific interactions involving SR proteins. DEK is an ~45-kD phosphoprotein that was previously identified in the context of several human diseases. It was first isolated as part of a fusion that arises in a subtype of acute myeloid leukemias (AMLs) involving (6;9) chromosomal translocations (von Lindern et al., 1992). These translocations occur within intron sequences of the genes encoding *dek* and the nucleoporin *can*, and result in the expression of an in-frame chimera encoding the majority of DEK fused NH_2-terminally to the COOH-terminal two-thirds of CAN. DEK was later identified as an autoimmune antigen in patients with pauciarticular onset juvenile rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases (Szer et al., 1994; Dong et al., 1998; Wichmann et al., 2000). In another study, it was reported that DEK binds to a TG-rich element, the pets (peri-ets) site, which is adjacent to two binding sites of the ets family member, Elf-1, in the HIV-2 enhancer, which mediates enhancer activation by a number of signaling processes (Fu et al., 1997). However, the specificity and functional significance of the binding of DEK to the pets site is not known. The results in the present study indicate that DEK may have one or more functions in association with pre-mRNA processing. In particular, the results identify DEK as one of the first factors that associates with mRNA in a splicing-dependent manner, indicating that it could function to coordinate splicing with one or more subsequent steps in gene expression.

Materials and Methods

Protein Purification

Fractionation of p130. Fractionation was initiated with ~200 ml of HeLa nuclear extract (~10 mg/ml) that was prepared as described by Dignam et al. (1983), except that the final dialysis was against a modified buffer D containing 10% glycerol (20 mM Hepes, pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM DTT). The dialyzed nuclear extract was chromatographed over a poly-U Sepharose column as described by Zamore and Green (1989). The flow-through fraction was chromatographed over a phosphocellulose column as described by Samuels et al. (1982). p130 and other SR proteins were detected entirely in the 1.0 M salt elution. The 1.0 M salt elution from the phosphocellulose column was dialyzed against SR-dialysis buffer (SRDB; 65 mM KCl, 15 mM NaCl, 10 mM Hepes, pH 7.6, 1 mM EDTA, 2 mM DTT, 5 mM KF, 5 mM β-glycerophosphate, 0.2 mM Pefabloc [Boehringer]; modified from Zahler et al., 1992). Precipitate formed during dialysis, which contained the majority of SRm160, but not p130, was removed by centrifugation. The cleared fraction was incubated for 15 min at 30°C in the presence of 1.5 mM ATP and 5 mM creatine-PO_4 to regenerate phosphoepitopes detected by mAb-104, and was then recentrifuged to remove further precipitate. The supernatant fraction was mixed with an equal volume of SRDB containing 6 M urea and then chromatographed over a Q-Sepharose column preequilibrated with 3 M urea-SRDB. Proteins were step-eluted with increasing concentrations of KCl in the 3 M urea-SRDB buffer. A sample of the

0.3 M step elution (Q-Sepharose-0.3 M, Q-0.3M), which contained the majority of p130 and other SR proteins detected by immunoblotting with mAb-104, was precipitated with TCA in the presence of deoxycholate. The TCA pellet was washed with ice-cold acetone, dried briefly, and resuspended in Laemmli loading buffer. The sample was separated on a 12% SDS polyacrylamide gel and detected by Coomassie staining (Fig. 1 C). The p130 doublet and other visible bands were excised, digested in situ with trypsin, and analyzed by MALDI-TOF mass spectroscopy using a PerSeptive Biosystems (model DE-STR) mass spectrometer. Details of sample preparation can be obtained from borealis biosciences, inc.

Purification of SR Family Proteins. Purification of the set of 6 defined SR proteins was performed essentially as described by Zahler et al. (1992).

Antibodies

The following antibodies were used in this study: murine mAb-104 (Roth et al., 1990); mAb-B1C8 (Blencowe et al., 1994; Wan et al., 1994; Matritech); mAb-B3 (Mortillaro et al., 1996); mAb-NM4 (Blencowe et al., 1995; Matritech); mAb anti-Flag (VWR-Scientific); rabbit polyclonal antibody (rAb)-DEK (Fornerod et al., 1995); and rAb-SRm300 (Blencowe et al., 2000).

Nuclear and Cytoplasmic Extracts

HeLa nuclear and cytoplasmic S100 extracts were prepared essentially as described by Dignam et al. (1983). Nuclear extract depleted of DEK protein was prepared by the same procedure as described for SRm160/300 in Blencowe et al. (1998).

Splicing and Immunoprecipitation Assays

Immunoprecipitation of SR protein complexes in the presence and absence of ribonuclease (Fig. 2) was performed as described by Eldridge et al. (1999). Immunoprecipitation of splicing complexes was performed as described in Blencowe et al. (1994). Splicing assays using the PIP85A pre-mRNA were carried out as described by Blencowe et al. (1998). Splicing assays using the dsx$(GAA)_6$ pre-mRNA were performed as described by Eldridge et al. (1999). The splicing reactions containing S100 extract (see Fig. 5) were performed in a total volume of 15 μl and were supplemented with ~3 μg of purified SR family proteins where indicated. The splicing reactions containing PIP85A pre-mRNA were analyzed on 15% denaturing polyacrylamide gels; doublesex (dsx) splicing reactions were analyzed on 7 or 10% denaturing polyacrylamide gels.

Affinity Selection of Splicing Complexes

Affinity selection of splicing complexes assembled on biotinylated dsx pre-mRNAs was performed essentially as described in Li and Blencowe (1999).

SDS-PAGE and Immunoblot Analysis

SDS-PAGE and immunoblots were performed essentially as described by Harlow and Lane (1988). Immunoblots in Figs. 1, 4, and 5 were developed using a secondary antibody conjugated to HRP and chemiluminescence detection (NEN Life Science Products), as per the manufacturer's instructions.

Immunocytochemistry

HeLa cells were immunolabeled as described by Blencowe et al. (1998). The samples were examined and recorded using a Leica-SP confocal microscope.

Results

DEK Copurifies with SR Proteins

To identify new SR-related proteins and associated factors that function in pre-mRNA processing, we have fractionated HeLa cell nuclear extract for proteins that react with mAb-104, which binds to a phosphoepitope located within the RS domains of many SR proteins (Roth et al., 1990).

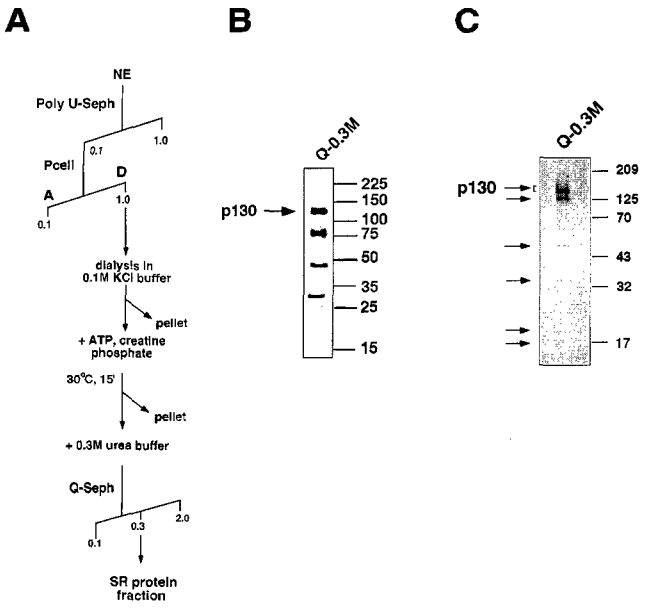

Figure 1. DEK copurifies with SR proteins. A, Fractionation scheme for a 130-kD antigen recognized by mAb-104 (p130; see Materials and Methods). B, mAb-104 immunoblot of the Q–Sepharose 0.3-M (Q-0.3M) fraction obtained by the scheme in A. SR proteins of ~75, 50, and 30 kD detected by mAb-104 copurify with p130. Size markers are indicated in kD. C, Coomassie-stained SDS polyacrylamide gel of the Q-0.3M fraction. The identities of the abundant polypeptides, migrating at 130, 50, and 34 kD, were identified by MALDI-TOF mass spectroscopy as Hel117, DEK, and SC35, respectively. Size markers are indicated in kD.

We initially focused our attention on an antigen of 130 kD (p130), since an SR protein of this size recognized by mAb-104 had not been isolated previously. Using immunoblotting with mAb-104 to monitor purification, we fractionated HeLa nuclear extract for p130 by the scheme shown in Fig. 1 A (see Materials and Methods). The 0.3 M KCl elution step from the final (Q-Sepharose) column yielded subfractions that were highly enriched in a doublet of mAb-104 antigens that migrated at 130 kD, as well as antigens detected by this antibody corresponding in size to previously identified SR family proteins of ~75, 55, and 30 kD. The levels of the p130 doublet detected by immunoblotting with mAb-104 correlated precisely with the levels of a doublet of polypeptides of 130 kD detected in the same subfractions by Coomassie staining, indicating that one or both of the latter species correspond to p130 (data not shown). Fig. 1 B shows an immunoblot of the peak Q-0.3M fraction probed with mAb-104, and Fig. 1 C shows a corresponding Coomassie-stained gel. Besides the 130-kD doublet, relatively abundant polypeptides of 120, 50, 35, 20, and 17 kD were detected in the fraction by Coomassie staining (indicated by arrows in Fig. 1 C).

To determine the identity of the p130 proteins, as well as the other proteins in the Q-0.3M fraction, the bands indicated in Fig. 1 C were excised, digested with trypsin in situ, and the released peptides were analyzed by MALDI-TOF mass spectroscopy. The masses of peptides from each protein were used to search for corresponding peptide masses in conceptually translated sequences from the databases.

Close matches were obtained between peptides from both polypeptides in the p130 doublet, identifying these as Hel117. The ~50- and 35-kD proteins were identified as DEK and SC35, respectively (data not shown). Consistent with its reactivity with mAb-104, Hel117 is a previously reported protein of unknown function that contains an RS domain (Sukegawa and Blobel, 1995), and SC35 is a previously characterized member of the SR family of splicing factors (Fu and Maniatis, 1992). Hel117 contains other domain features found in splicing factors, including a DEAD-box and associated motifs shared between proteins with dsRNA unwinding and/or nucleic acid-dependent ATPase activity. It also contains a separate domain that is homologous to a region within the yeast splicing factor, PRP5. Consistent with a possible role in splicing, Hel117 was previously localized to nuclear speckle domains enriched in pre-mRNA splicing factors (Sukegawa and Blobel, 1995), and we have found that it associates with other SR proteins and splicing complexes in vitro (unpublished observations). Interestingly, DEK is a multidisease-associated phosphoprotein of unknown function that was originally identified as an in-frame fusion partner with the nucleoporin CAN, generated by a specific (6;9) chromosomal translocation in a subset of AML (von Lindern et al., 1992; see Introduction). It contains several regions rich in charged residues, including an NH_2-terminal acidic domain similar to those found in transcriptional activator proteins. A comparison of the DEK amino acid sequence against sequences in the databases identified homologous sequences within predicted open reading frames of zebrafish, fly, and plant expressed sequence tag cDNAs, but did not reveal a significant homology with any characterized protein (data not shown).

The 55- and 75-kD SR proteins that were detected in the Q-0.3M fraction by immunoblotting with mAb-104 were not detected in the Coomassie stained gel in Fig. 1 C because these proteins are lower in abundance than the polypeptides that were analyzed. The detection of these antigens and the identification by mass spectroscopy of two RS domain proteins in the Q-0.3M fraction, Hel117 and SC35, demonstrates that the fractionation scheme in Fig. 1 A enriches for SR proteins associated with pre-mRNA processing. The cofractionation of DEK with several SR proteins suggested that it either has similar chromatographic properties as these factors, or, more interestingly, that it may be associated with one or more SR proteins in a complex. To distinguish between these possibilities, we next addressed whether DEK interacts with SR proteins.

DEK Associates with SR Proteins In Vitro

Immunoprecipitates were collected from HeLa nuclear extract using an mAb specific for the SRm160 subunit of the SRm160/300 splicing coactivator (mAb-B1C8; Blencowe et al., 1994; Wan et al., 1994). This antibody immunoprecipitates SRm160/300 efficiently and, in the presence of low salt concentrations, coimmunoprecipitates several proteins that contain RS domains, including Hel117, hTra2beta (a human homologue of the *Drosophila melanogaster* alternative splicing factor, Tra2) and SR family proteins (Eldridge et al., 1999; and our unpublished data).

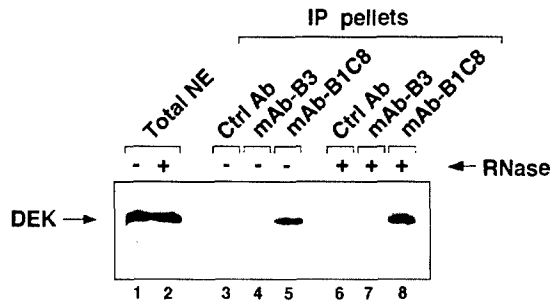

Figure 2. DEK associates with SR proteins. Immunoprecipitates were collected from HeLa nuclear extract preincubated with (lanes 6–8) or without (lanes 3–5) RNase using the following antibodies: an mAb specific for the SRm160 subunit of the SRm160/300 splicing coactivator (mAb-B1C8, lanes 5 and 8); an mAb specific for hyperphosphorylated RNA pol II large subunit (mAb-B3, lanes 4 and 7); and a control antiserum (rAb anti-mouse, lanes 3 and 6). The immunoprecipitates were separated on an SDS polyacrylamide gel and immunoblotted with rAb-DEK. The corresponding total HeLa nuclear extracts treated without or with RNase, corresponding to 10% of the amount of nuclear extract used in each immunoprecipitation, are separated in lanes 1 and 2, respectively.

To determine whether DEK associates with one or more of these SR proteins, the immunoprecipitates were immunoblotted with an antibody that is highly specific for DEK (rAb-DEK; Fig. 2; Fornerod et al., 1995). In total HeLa nuclear extract, rAb-DEK detected a major band of ~50-55 kD and a minor band of ~40 kD. The major band corresponds in size to a form of DEK that is posttranslationally modified by phosphorylation, whereas the minor band is thought to correspond to a cleavage product of DEK (Fig. 2, lanes 1 and 2; Fornerod et al., 1995). The specificity of rAb-DEK for DEK has been confirmed by immunoblotting whole cell extracts from $DEK^{+/+}$ and $DEK^{-/-}$ mice. Both bands are detected by rAb-DEK in +/+ extracts, but neither band, nor any other antigen, is detected in the −/− extracts (data not shown).

mAb-B1C8, but not a control mAb of the same isotype that is specific for a hyperphosphorylated form of the RNA pol II large subunit (mAb-B3; Mortillaro et al., 1996), specifically coimmunoprecipitated DEK (Fig. 2, compare lane 5 with 4). This coimmunoprecipitation was not reduced by extensive pretreatment of nuclear extract with RNAse (Fig. 2, compare lanes 5 and 8), indicating that DEK associates with one or more SR proteins through protein–protein interactions and is not tethered to these proteins by RNA. Thus, consistent with the cofractionation of DEK with SR proteins (Fig. 1), the results indicate that DEK is associated with one or more SR proteins that function in pre-mRNA splicing. To extend these observations, we next determined whether or not DEK associates with SR proteins during the formation of pre-mRNA splicing complexes (Fig. 3).

Association of DEK with Splicing Complexes

Remarkably, rAb-DEK immunoprecipitated splicing complexes assembled on a constitutively spliced pre-mRNA derived from adenovirus PIP85A (Fig. 3 A). Similar to a

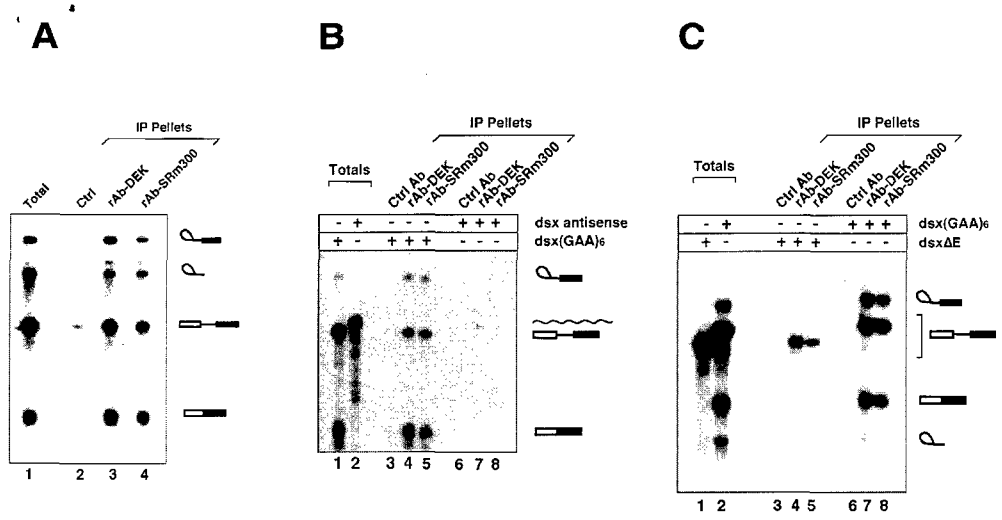

Figure 3. Association of DEK with splicing complexes. A, Immunoprecipitation of splicing complexes with rAb-DEK from reactions incubated for 1 h containing PIP85A pre-mRNA. RNA recovered after immunoprecipitation (lanes 2–4) and RNA recovered directly from a parallel splicing reaction (lane 1), was loaded on a 15% denaturing polyacrylamide urea gel. RNA loaded in lane 1 represents 25% of the total amount recovered, whereas RNA loaded in lanes 2–4 from each immunoprecipitation represents 50% of the total amounts recovered. Immunoprecipitations were performed with preimmune serum (lane 2), rAb-DEK (lane 3), and rAb-SRm300 as a positive control (lane 4). B, Complexes were immunoprecipitated from splicing reactions incubated for 45 min containing either a dsx$(GAA)_6$ pre-mRNA (lanes 1, and 3–5) or the corresponding antisense RNA (lanes 2, and 6–8). RNA recovered after immunoprecipitation (lanes 3–8) and RNA recovered directly from parallel splicing reactions (lanes 1 and 2) was loaded on a 10% denaturing polyacrylamide urea gel. The antibodies used for immunoprecipitation and the relative amounts of RNA loaded from the total and immunoprecipitation samples are as described in A. C, Association of DEK with a two-exon dsx pre-mRNA is promoted by a 6xGAA ESE sequence in the 3′ exon. Immunoprecipitation was performed from splicing reactions incubated for 45 min containing dsx pre-mRNA lacking an ESE (dsxΔE; lanes 1, and 3–5) or from reactions containing dsx pre-mRNA with the 6xGAA ESE (dsx$(GAA)_6$; lanes 2, and 6–8). RNA recovered after immunoprecipitation (lanes 3–8) and RNA recovered directly from a parallel splicing reactions (lanes 1 and 2) was loaded on a 10% denaturing polyacrylamide urea gel. Antibodies used for immunoprecipitation and the relative amounts of RNA loaded from the total and immunoprecipitation samples are as described in A.

polyclonal antibody specific for SRm300 (rAb-SRm300; Blencowe et al., 2000), which was used as a positive control for immunoprecipitation, rAb-DEK efficiently immunoprecipitated splicing complexes containing exon sequences (pre-mRNA, splicing intermediates, and exon-product RNA) and, to a lesser degree, the complex containing the lariat product of the splicing reaction (Fig. 3 A, lanes 3 and 4). The immunoprecipitation of splicing complexes by rAb-DEK was specific since neither preimmune serum, nor a saturating amount of a control immune serum bound to protein A beads, immunoprecipitated splicing complexes (Fig. 3 A, lane 2, and data not shown; see also Eldridge et al., 1999). These results provide evidence that DEK associates with splicing complexes through both steps of the splicing reaction. Moreover, the similar profile of immunoprecipitation of splicing complexes observed between rAb-DEK and rAb-SRm300 is consistent with the data in Fig. 2, indicating that DEK and SRm160/300 associate with each other (see below).

To investigate the specificity of the interaction of DEK with splicing complexes in more detail, we next determined whether it can associate with a distinct pre-mRNA, and also whether it can associate with an RNA that is not a splicing substrate. To test for these properties, rAb-DEK was assayed for its ability to immunoprecipitate splicing complexes assembled on a pre-mRNA derived from exons 3 and 4 of the *dsx* gene of *Drosophila*, and also the corresponding antisense RNA, which does not assemble functional splicing complexes (Fig. 3 B). Wild-type *dsx* pre-mRNA contains a suboptimal 3′ splice site upstream of exon 4. In *Drosophila*, efficient recognition of this splice site and subsequent intron removal requires the assembly on a specialized exonic splicing enhancer (ESE) within exon 4 of a multisubunit complex that contains the RS domain splicing factors Tra, Tra2, and the SR family protein RBP-1 (SRp20; Inoue et al., 1992; Tian and Maniatis, 1993, 1994). The substrate used in the present study contains a typical purine-rich mammalian ESE consisting of six GAA repeats in place of the dsx ESE (Yeakley et al., 1996). This 6xGAA ESE promotes the stable association of hTra2β, several SR family proteins, and SRm160/300 with the dsx pre-mRNA, and is required for efficient splicing of this substrate in HeLa nuclear extracts (see below; Yeakley et al., 1996; Eldridge et al., 1999; Li and Blencowe, 1999). The results in Fig. 3 B demonstrate that rAb-DEK, like rAb-SRm300, efficiently immunoprecipitated splicing complexes assembled on the dsx$(GAA)_6$ pre-mRNA (lanes 4 and 5), but only weakly immunoprecipitated the antisense RNA (lanes 7 and 8). These results indicate that DEK may be a general component of splicing complexes, but that it does not associate stably with an RNA that does not form functional splicing complexes.

Given the association of DEK with SR proteins, including SRm160/300 observed in Fig. 2, and the similar profiles of immunoprecipitation of splicing complexes observed between antibodies to both DEK and SRm300 (Fig. 3, A and B), we next determined whether or not SR proteins mediate the association of DEK with splicing complexes. To test if this is the case, we initially compared the ability of DEK to associate with splicing complexes assembled on the dsx$(GAA)_6$ pre-mRNA versus the same substrate lacking an ESE (dsxΔE; Fig. 3 C). Previously, we have

shown that U1 snRNP and U2AF-65 kD bind equally to these substrates but, as mentioned above, SRm160/300, Tra2beta, and SR family proteins only associate stably with the dsx(GAA)$_6$ pre-mRNA (Eldridge et al., 1999; Li and Blencowe, 1999). Similar to rAb-SRm300, rAb-DEK efficiently immunoprecipitated splicing complexes assembled on the dsx(GAA)$_6$ substrate, but inefficiently immunoprecipitated complexes assembled on the dsxΔE substrate (Fig. 3 C, compare lanes 7 and 8 with 4 and 5). The data demonstrate that a 6xGAA ESE results in a significant increase in the association of both DEK and SRm300 with the dsx pre-mRNA, again consistent with the data in Fig. 2 indicating that these components interact. As before, the ability of rAb-DEK and rAb-SRm300 to immunoprecipitate splicing complexes on the dsx substrates was not due to nonspecific interactions since neither preimmune serum, nor high levels of control immune sera, immunoprecipitated significant levels of these complexes (Fig. 3 C, lanes 3 and 6; see also Eldridge et al., 1999).

To confirm that the different levels of immunoprecipitation of the dsxΔE and dsx(GAA)$_6$ splicing complexes by rAb-DEK reflect the differential association of DEK with these substrates and are not due to the differential accessibility to antibody in these complexes, we next probed directly for the presence of DEK in splicing complexes affinity-selected on the dsx substrates (Fig. 4). Splicing complexes were assembled on biotinylated derivatives of the dsx pre-mRNAs and affinity-selected using streptavidin agarose. Bound proteins were eluted in high salt and immunoblotted first with mAb-104 to confirm the recovery of splicing complex proteins (Fig. 4 A). In agreement with our previous results (Li and Blencowe, 1999), low levels of SR proteins of 30, 40, 55, and 75 kD were detected by mAb-104 in splicing complexes affinity-selected on the dsxΔE pre-mRNA (Fig. 4 A, lane 3), whereas the levels of these proteins increased significantly on the dsx(GAA)$_6$ pre-mRNA (Fig. 4 A, lane 4). Consistent with the immunoprecipitation results in Fig. 3, DEK was detected in splicing complexes assembled on the dsx(GAA)$_6$ substrate, but not in complexes on the dsxΔE pre-mRNA (Fig. 4 B, compare lanes 3 and 4). The detection of DEK in association with the dsx(GAA)$_6$ substrate was specific, since essentially no DEK was selected from a splicing reaction containing complexes assembled on a dsx(GAA)$_6$ pre-mRNA lacking biotinylated residues (Fig. 4 B, lane 2). The absence of a level of DEK above background in the affinity-selected dsxΔE complexes (Fig. 4 B, lane 3), in comparison with the low level detected in association with the dsxΔE pre-mRNA by immunoprecipitation (Fig. 3 C, lane 4), is probably due to the more stringent conditions used in the affinity-selection assay (300 mM salt) compared with the conditions in the immunoprecipitation assay (100 mM salt; see Materials and Methods). Moreover, the higher levels of SR proteins detected in the affinity-selected splicing complexes compared with DEK indicates that they may be more stably associated with the dsx pre-mRNA (Fig. 4, A and B, compare lanes 1, 3, and 4). These results confirm that DEK is associated with splicing complexes on the dsx(GAA)$_6$ pre-mRNA and that its association with this substrate, like SRm160/300, hTra2beta, and SR family proteins, is promoted by a purine-rich ESE.

To confirm that SR proteins are required for the stable

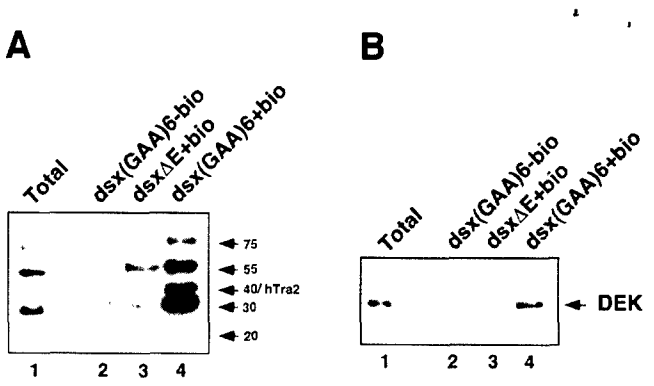

Figure 4. Detection of DEK in affinity-purified splicing complexes. A and B, Biotinylated dsx pre-mRNAs were transcribed cold and incubated in splicing reactions for 15 min before selection on streptavidin-agarose. Selections were performed from splicing reactions incubated with a control, non-biotinylated, dsx pre-mRNA (dsx(GAA)$_6$−bio, lane 2), with a biotinylated dsx pre-mRNA lacking an ESE (dsxΔE+bio, lane 3), and with a biotinylated dsx pre-mRNA containing a 6xGAA ESE (dsx(GAA)$_6$+bio, lane 4). Proteins recovered from the affinity-selected splicing complexes were separated on 12% SDS polyacrylamide gels and analyzed by immunoblotting with mAb-104 (A) and rAb-DEK (B). Total nuclear extract (lane 1), corresponding to ~3% of the amount of extract used in each affinity selection, was separated as a marker for the eluted SR and DEK proteins. Sizes of the SR proteins (in kD) detected by mAb-104 are indicated to the right of A.

association of DEK with pre-mRNA, we next assayed the ability of rAb-DEK to immunoprecipitate splicing complexes from a cytoplasmic S100 extract (Fig. 5). S100 extracts contain all of the factors required for splicing, except SR proteins (Krainer et al., 1990b). These extracts contain a lower level of DEK than in the same volume of HeLa nuclear extract (data not shown). Addition of purified SR family proteins to an S100 reaction resulted in a partial restoration of splicing activity to the PIP85A pre-mRNA (Fig. 5, compare lanes 1 and 2). The rAb-DEK antibody immunoprecipitated a low level of PIP85A pre-mRNA from an S100 reaction in the absence of SR family proteins (Fig. 5, lane 4). However, upon addition of purified SR family proteins, immunoprecipitation of PIP85A pre-mRNA by rAb-DEK was restored to a level comparable to that observed in reactions containing total nuclear extract (Fig. 5, lane 5, and data not shown). This promotion of immunoprecipitation by SR proteins was not due to an increase in the level of DEK in the splicing reaction, since no DEK was detected in the purified SR protein preparation by immunoblotting with rAb-DEK (Fig. 5 B, lane 3). Moreover, no difference in the level of DEK was observed in the S100 splicing reactions after incubation with or without SR proteins (Fig. 5 B, compare lanes 1 and 2). Together with the data in Figs. 3 and 4, these results demonstrate that the association of DEK with pre-mRNA requires interactions mediated by SR proteins.

DEK Associates with SR Proteins In Vivo

To investigate whether DEK associates with splicing components in vivo, we double-immunolabeled HeLa cells with rAb-DEK and the anti-SR protein antibody, mAb-

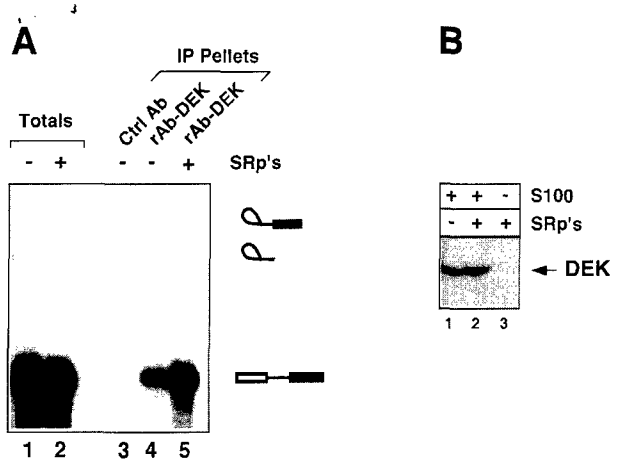

Figure 5. SR proteins promote the association of DEK with pre-mRNA in S100 splicing reactions. A, Immunoprecipitations with rAb-DEK were performed from S100 splicing reactions incubated for 1 h containing PIP85A pre-mRNA, with (lanes 2 and 5) or without (lanes 1, 3, and 4) purified SR family proteins. RNA recovered after immunoprecipitation (lanes 3–5) and RNA recovered directly from a parallel splicing reaction (lanes 1 and 2) was loaded on a 15% denaturing polyacrylamide urea gel. RNA loaded in lane 1 represents 25% of the total amount recovered, whereas RNA loaded in lanes 2–4 from each immunoprecipitation represents 50% of the total amounts recovered. Immunoprecipitations were performed with a preimmune serum (lane 3) and rAb-DEK (lanes 4 and 5). B, Proteins recovered from parallel S100 splicing reactions as shown in A were separated on a 12% SDS polyacrylamide gel and immunoblotted with rAb-DEK. Lanes 1 and 2 correspond to lanes 1 and 2 in A. Lane 3 contains ~3 μg of the SR protein preparation used in A.

NM4 (Blencowe et al., 1995; Fig. 6, A–F). Similar to many antibodies that recognize SR proteins, mAb-NM4 specifically immunolabels nucleoplasmic interchromatin granule clusters or speckles that are highly enriched in splicing factors (B and E, and data not shown). In the majority of cells within a field, rAb-DEK labeling reveals that DEK is specifically localized in the nucleus in a diffuse-granular pattern that largely, but not completely, excludes nucleoli (Fig. 6 A). In these cells, DEK overlaps with speckle domains, although it does not appear significantly more concentrated in these structures than in other regions of the nucleoplasm (Fig. 6 C). However, in ~15% of cells within a field, DEK is significantly more enriched in speckle structures compared with the surrounding nucleoplasm (Fig. 6, compare D–F with A–C). The detection of DEK by immunostaining with rAb-DEK is specific since preimmune serum did not immunolabel nuclei (Fornerod et al., 1996; data not shown). These results indicate that in a subset of cells, DEK is concentrated with SR proteins and other splicing factors in nuclear speckle structures.

To extend these observations, we next tested whether the localization of DEK is influenced by the intranuclear distribution of SR proteins. Recently, we have observed that SRm160 containing an NH_2-terminal Flag epitope (Flag-SRm160), when overexpressed by transient transfection, concentrates with endogenous SR proteins in enlarged speckle structures (Fig. 6 H, and data not shown). To determine whether the elevated levels of SRm160 in speckles results in a change in the nucleoplasmic distribution of DEK, cells transiently transfected with a flag-SRm160 construct were double-immunolabeled with anti-flag and rAb-DEK antibodies. Significantly, the presence of flag-SRm160 in speckles (Fig. 6 H) correlated with an accumulation of DEK in these structures (Fig. 6 G and I). This effect was specific since the distribution of DEK was not altered significantly by the overexpression of a flag-B23 protein, which concentrates in nucleoli (Fig. 6, compare J–L). Although every speckle containing flag-SRm160 also contained an increased level of DEK, in some cases the size of the speckle domain detected with the antiflag antibody was significantly smaller than the size of the corresponding speckle domain detected with rAb-DEK. This may reflect differences in the relative concentrations of these proteins between speckles, and/or the differential accessibility of these proteins to antibodies in different speckles. In either case, the results demonstrate that the distribution of DEK within nuclei can be specifically influenced by the localization of SRm160, and that, consistent with the in vitro interactions with SR proteins, including SRm160/300 described above, DEK probably concentrates in speckles by associating with one or more of these splicing factors.

Depletion of DEK Does Not Prevent Pre-mRNA Splicing

The results so far suggest that DEK could function as a splicing factor, perhaps by associating with one or more SR proteins and modulating their activity. To investigate this possibility, we have prepared nuclear extracts that are specifically and efficiently depleted of DEK (Fig. 7 A, compare lanes 1 and 2). Surprisingly, efficient immunodepletion of DEK did not affect the level of splicing of several pre-mRNAs tested, including the PIP85A (Fig. 7 B, compare lanes 1 and 2) and the dsx$(GAA)_6$ substrate (data not shown). Likewise, in contrast to SR proteins and heteronucleoriboprotein (hnRNP) A1, which promote the selection of intron proximal and distal 5′ splice sites, respectively (Ge and Manley, 1990; Krainer et al., 1990a; Fu et al., 1992; Mayeda and Krainer, 1992; Cáceres et al., 1994), altering the level of DEK by specific immunodepletion in vitro, or by overexpression in vivo, did not significantly alter the ratio of intron distal to proximal splice sites in different pre-mRNAs containing cis-competing splice sites (data not shown). Whereas it cannot be excluded that a minor amount of DEK remaining in the immunodepleted extract is important for splicing activity, or that DEK is important for the splicing of specific substrates not assayed, the results so far suggest that DEK may provide a function that is not directly related to splicing activity.

The Association of DEK with the Exon-product RNA Requires Prior Splicing

The experiments in Fig. 3 reveal that DEK, like SRm160/300, remains bound preferentially to spliced exons after splicing. This observation, and the finding that DEK does not associate efficiently with RNAs that cannot assemble functional splicing complexes, raises the possibility that its association with spliced exons requires the prior formation of a spliceosome. To determine whether the association of

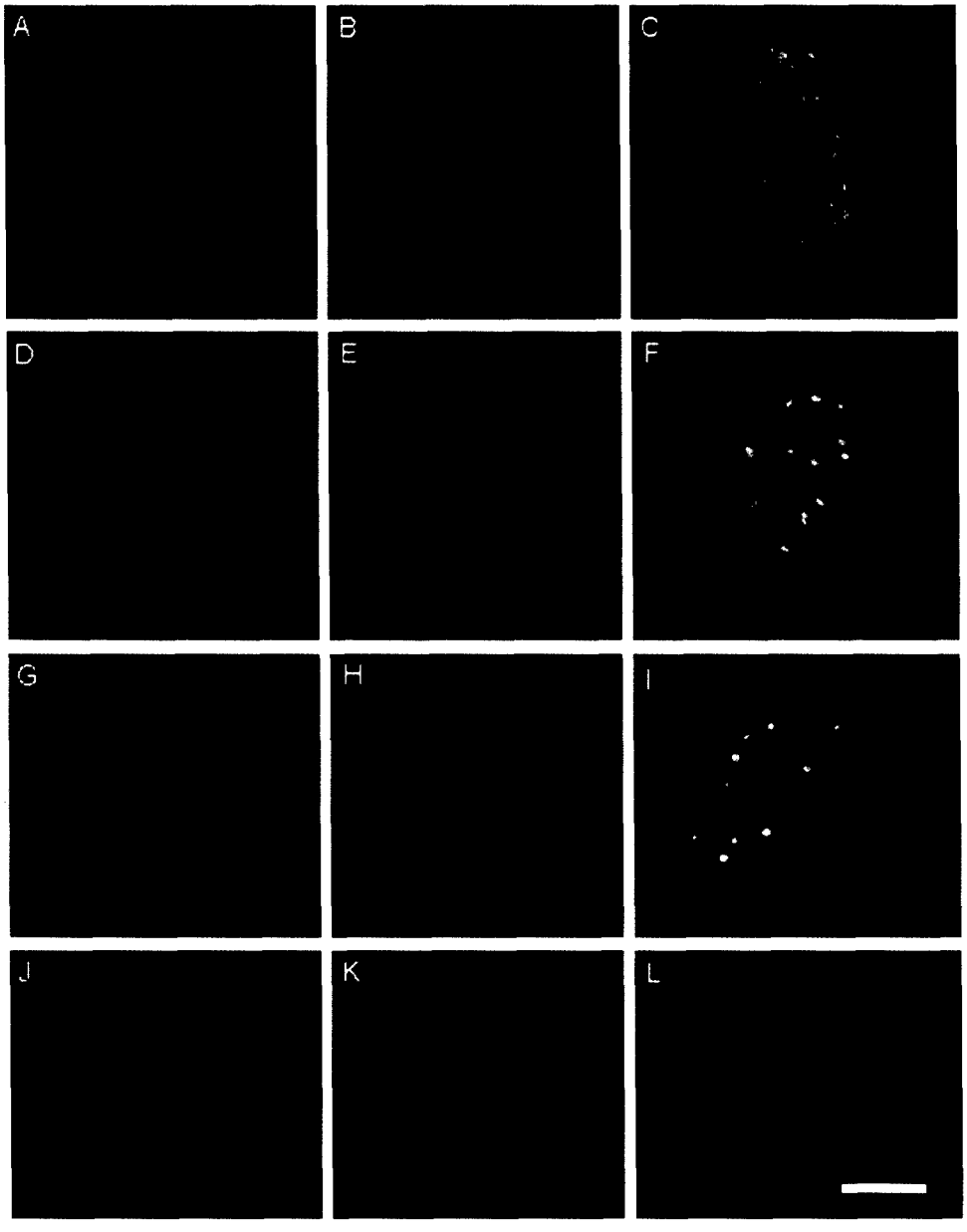

Figure 6. Association of DEK with splicing components in vivo. A–F. HeLa cells were immunolabeled with rAb-DEK (red; A and D) and the anti-SR protein antibody, mAb-NM4 (green; B and E). In the majority of cells, DEK is diffusely distributed throughout the nucleoplasm, partially excluding nucleoli (A). In a subset of cells (~15%), DEK is detected at higher levels in interchromatin granule clusters containing SR proteins compared with the surrounding nucleoplasm (compare D and A, and the corresponding overlays between D and E, and A and B in F and C, respectively). G–L. Cells were transfected with constructs expressing NH_2-terminal flag-tagged SRm160 (flag-SRm160; G–I) or NH_2-terminal flag-tagged B23 (flag-B23; J–L), and the effects on overexpression of these proteins on the distribution of DEK were examined. The transfected cells were double-immunolabeled with rAb-DEK (red; G and J) and antiflag (H and K) antibodies; the sites of overlap between the signals in G and H, and J and K, are shown in the overlays (I and L, respectively). All of the images were scanned using a Leica SP confocal microscope. Bar, 5 μm).

DEK with spliced exons is facilitated by splicing, we compared the ability of rAb-DEK to immunoprecipitate complexes formed on the exon-product RNA generated by splicing of PIP85A pre-mRNA, versus complexes formed on the identical exon-product RNA that has been derived by transcription from a PIP85A cDNA construct (Fig. 8). In this assay, splicing reactions were incubated with equal amounts of PIP85A pre-mRNA and a second RNA that is not a splicing substrate (a fragment of the HIV-1 Rev-response element), as an internal control RNA for recovery (Fig. 8, lanes 1 and 3). Although this latter RNA does not assemble into functional splicing complexes, like the dsx antisense and dsxΔE transcripts, it associates weakly with SRm160/300 and DEK, permitting its use for monitoring immunoprecipitation levels (see below; data not shown). In a parallel reaction, an equivalent amount of the internal control RNA was mixed with an exon-product RNA transcribed from the PIP85 cDNA construct (Fig. 8, lanes 2 and 4). Both reactions were incubated for one hour under splicing conditions before immunoprecipitation with rAb-DEK. Significantly, although a higher level of cDNA-derived exon-product RNA than spliceosome-derived exon-product RNA is present in the total splicing reactions (Fig. 8, compare lanes 1 and 3 with lanes 2 and 4), rAb-DEK only immunoprecipitated efficiently the spliceosome-derived exon-product RNA (compare lanes 7 and 8). This difference was not due to a nonspecific loss of the cDNA-derived exon-product RNA, since approximately equivalent levels of the internal control RNA were immunoprecipitated from both reactions (Fig. 8, compare lanes 7 and 8). As observed earlier (Fig. 3), in contrast to the efficient immunoprecipitation of the spliceosome-derived exon-product complex, rAb-DEK immunoprecipitated a significantly lower level of the lariat-product complex of the splicing reaction. This is consistent with the observation that the majority (80–95%) of exon-product

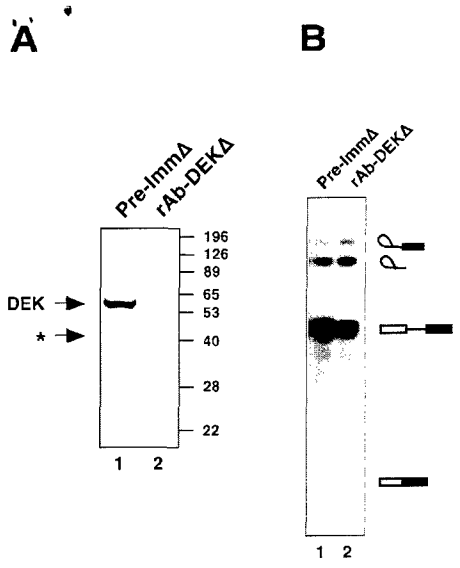

Figure 7. Immunodepletion of DEK does not prevent pre-mRNA splicing. A, HeLa nuclear extracts immunodepleted of DEK with rAb-DEK (lane 2) or mock-depleted with preimmune serum (lane 1) were separated on a 12% SDS polyacrylamide gel and immunoblotted with rAb-DEK. Size markers are indicated in kD. B, Splicing reactions containing rAb-DEK or preimmune-depleted nuclear extract were incubated with PIP85A pre-mRNA for 1 h. RNA recovered from the splicing reactions was separated on a 15% denaturing polyacrylamide gel.

RNAs generated by splicing are released from the spliceosome during in vitro splicing reactions (Le Hir et al., 2000), and also that a relatively low level of DEK remains bound to the lariat-product complex after splicing. Preferential immunoprecipitation of the PIP85A exon-product complex that had been generated by splicing, over the complex formed on the cDNA-derived exon-product RNA, was also observed with antibodies to both subunits of the SRm160/300 splicing coactivator (unpublished observations). This is in agreement with very recent results showing that SRm160 specifically cross-links to spliced exons dependent on prior splicing (Le Hir et al., 2000; see Discussion), and also our finding in the present study that DEK and SRm160/300 associate. Thus, DEK forms a component of an exon-product complex, the assembly of which requires prior splicing. These results suggest that DEK could function to tag spliced mRNAs as a means to facilitate one or more postsplicing processes.

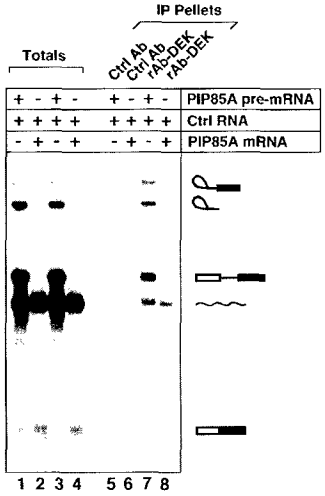

Figure 8. Association of DEK with spliced exons requires prior splicing. Immunoprecipitations were performed with rAb-DEK from splicing reactions incubated for 1 h containing PIP85A pre-mRNA or a PIP85A exon-product RNA transcribed from a PIP85A cDNA construct. Both reactions also contained an internal control RNA for recovery (indicated by a wavy line; see Results). RNA recovered after immunoprecipitation with rAb-DEK (lanes 7 and 8) or control serum (lanes 5 and 6) was separated, along with the corresponding total samples (lanes 3 and 4, and 1 and 2, respectively), on a 15% denaturing polyacrylamide gel.

Discussion

We have identified the AML-associated protein DEK as a factor that interacts in vitro and in vivo with SR proteins involved in pre-mRNA splicing. DEK associates efficiently with splicing complexes through interactions mediated by SR proteins. Its association with a pre-mRNA is stimulated by the presence of an ESE that binds to SR family and SR-related proteins. Moreover, its association with a pre-mRNA is stimulated by the addition of purified SR proteins to an SR protein-depleted reaction. After splicing, DEK remains associated preferentially with exon-product complexes. Importantly, this interaction requires prior splicing since DEK does not associate with an exon-product RNA that has not been generated by splicing. The results indicate that interactions involving DEK may distinguish a transcript that has been through splicing from a transcript that has not. Moreover, they suggest that DEK could function to communicate between splicing and one or more steps downstream in gene expression.

Increasing evidence indicates that the nuclear history of a transcript can influence its subsequent metabolism. For example, it has been demonstrated in many studies that the presence of an intron is important for efficient gene expression. These intron-mediated effects have been attributed to influences on mRNA stability, transport and translation. For example, prior splicing is important for translation-dependent degradation of mRNA by the nonsense-mediated decay (NMD) pathway (reviewed by Maquat, 1995, 1996; Hentze and Kulozik, 1999). In a recent study, it was shown that an exon-product RNA generated by splicing assembles into a complex that is significantly larger and more efficiently exported to the cytoplasm than the complex formed on the equivalent RNA synthesized from an intron-less transcript (Luo and Reed, 1999). The presence and location of an intron, whether at the 5′ or 3′ end of a transcript, can also influence its translation in the cytoplasm. For example, *Xenopus* maternal mRNAs transcribed in vivo are normally translationally masked, but can be relieved from translational repression by the insertion of an intron at the 5′, but not at the 3′ end, of a transcript (Matsumoto et al., 1998). All of the above observations indicate the existence of factors that package an mRNP dependent on its passage through the spliceosome and that modulate downstream steps in gene expression. However, the factors that specifically tag mRNPs dependent on prior splicing are not known.

hnRNPs such as hnRNP-A1 are candidate factors for influencing the composition and activity of an mRNP dependent on prior splicing. HnRNP-A1 associates with nascent pre-mRNA, influences splicing activity, and associates

with spliced mRNA in the nucleus and cytoplasm; it has also been detected in association with translationally engaged mRNA (Mayeda and Krainer, 1992; Visa et al., 1996). However, neither hnRNP-A1 or other hnRNP proteins have been detected in purified spliceosomes and it is also not known whether their association with mRNPs is dependent on prior splicing. In contrast, it has been demonstrated that specific SR proteins, including the SRm160/300 splicing coactivator subunits and ASF/SF2, associate with splicing complexes through both steps of the splicing reaction and remain preferentially bound to the exon-product RNA (Blencowe et al., 1998, 2000; Hanamura et al., 1998). Moreover, a *Chironomas tentans* SR family protein, hrp45, which is related to mammalian ASF/SF2, associates with intron-containing Balbiani ring transcripts and remains bound to spliced mRNP until the time the mRNP is translocated through the nuclear pore complex, upon which it appears to be released (Alzhanova-Ericsson et al., 1996). Very recently, Le Hir and colleagues have used an elegant cross-linking strategy to detect proteins that cross-link at or near spliced exon junctions, dependent on prior splicing. In this study, splicing-dependent cross-links were identified for SRm160, the U5 snRNP protein hPRP8/p220, and several unidentified proteins (Le Hir et al., 2000). The results in the present study are consistent with these findings. In particular, since the association of DEK with pre-mRNA is promoted by interactions with SR proteins, it is possible that its association with spliced exons is mediated by SRm160. The results further suggest that interactions involving factors that define mRNP composition occur as early as the commitment stage of splicing complex formation, when U1 snRNP and SR family proteins bind to the pre-mRNA and facilitate the association of factors including SRm160/300 and DEK with the substrate (Blencowe et al., 1998; present study).

The results of the present study, taken together with a previous finding indicating that DEK associates with the pets site within the HIV-II promoter (Fu et al., 1997; see Introduction), reveals an interesting relationship with a growing number of factors that are associated with both RNA processing and malignant transformation. For example, it has been reported that certain isoforms of the Wilm's tumor protein (WT-1) bind to DNA at specific promoter sequences and concentrate within transcription factor domains in vivo, whereas other isoforms specifically associate with splicing components, including snRNPs and the 65-kD subunit of the U2 snRNP auxiliary factor (U2AF-65), and concentrate within splicing factor domains in the nucleus (Larsson et al., 1995; Davies et al., 1998). In another case, the oncoprotein TLS/FUS, which becomes fused to the CHOP protein in liposarcomas, or to the ERG protein in a subset of myeloid leukemias, binds to both DNA and RNA and has recently been identified as hnRNP-P2, a component of hnRNP complexes (Calvio et al., 1995). Intriguingly, also similar to DEK, it has been reported that TLS/FUS interacts with SR protein splicing factors (Yang et al., 1998). Another factor with dual RNA and DNA binding properties is the Ets-related transcription factor Spi-1/PU.1. This factor, besides its role as a hematopoietic-specific transcription factor, has been reported to interact with both TLS/FUS and the polypyrimidine tract-binding protein NonO/p54nrb, and to influence splicing activity (Hallier et al., 1996, 1998). NonO/p54nrb, which also has the ability to bind to both DNA and RNA, and a closely related protein, PSF (the polypyrimidine binding protein–associated splicing factor), were both identified recently in fusions with the transcription factor TFE in papillary renal cell carcinomas (Clark et al., 1997). Thus, all of the RNA processing-associated factors implicated in different human malignancies to date, like DEK, have associations with transcription components, as well as with pre-mRNA processing. While the significance of these similar features is not known, it is tempting to speculate that malignancies involving alterations to DEK and these other proteins could affect functions in transcription, RNA processing, or perhaps interactions that coordinate these steps in gene expression (see Introduction). However, it is also possible that AMLs involving the DEK/CAN fusion involve an alteration to the normal function of CAN (Fornerod et al., 1995). Further work will be required to determine the normal function of DEK and whether its function is altered by fusion to CAN in AML.

The results in the present study suggest that DEK has a function in association with pre-mRNA metabolism. In particular, its association with spliced exons dependent on prior splicing represents one of the first examples of a protein with this property. It is possible that this splicing-dependent association is important for facilitating one or more steps downstream in gene expression that are influenced by the prior removal of an intron in pre-mRNA. Thus, based on the previous and present findings, DEK is a candidate for mediating interactions between transcription and pre-mRNA processing, as well as between splicing and one or more subsequent splicing-dependent steps in the gene expression pathway.

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Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases

Benjamin J. Blencowe

Exonic splicing enhancers (ESEs) are discrete sequences within exons that promote both constitutive and regulated splicing. The precise mechanism by which ESEs facilitate the assembly of splicing complexes has been controversial. However, recent studies have provided insights into this question and have led to a new model for ESE function. Other recent work has suggested that ESEs are comprised of diverse sequences and occur frequently within exons. Ominously, these latter studies predict that many human genetic diseases linked to mutations within exons might be caused by the inactivation of ESEs.

ONE OF THE most remarkable features of the mammalian pre-mRNA splicing machinery is its ability to select precisely correct pairs of splice sites among a myriad of potential, but inappropriate splice sites. This high degree of selectivity is particularly astounding given the short and poorly conserved nature of splice-site sequences in higher eukaryotes (see Fig. 1). The removal of intron sequences by splicing occurs by two sequential transesterification reaction steps that are catalysed by the components of a large (~60S) RNA–protein complex, termed the spliceosome (reviewed in Refs 1,2). The formation of the major spliceosome involves the step-wise assembly of four small nuclear ribonucleoprotein particles (snRNPs U1, U2, U4/U6 and U5) and many non-snRNP splicing factors on a pre-mRNA. A large number of non-snRNP splicing factors contain one or more domains rich in alternating arginine and serine residues, referred to as RS domains (see Fig. 2; reviewed in Refs 3,4). These include proteins of the SR family, each member of which contains one or two N-terminal RNA recognition motifs (RRMs) that function in sequence-specific RNA binding and a C-terminal RS domain that is required for protein–protein interactions with other RS domains.

SR-family proteins function in both constitutive and regulated pre-mRNA splicing. Other non-snRNP splicing factors that contain RS domains are structurally and functionally distinct from the SR family and are referred to as SR-related proteins (see Fig. 2; reviewed in Refs 3,5; and see below). SR-family and SR-related proteins function early in spliceosome formation to promote the formation of complexes containing U1 snRNP bound to the 5′ splice site and U2 snRNP bound to the pre-mRNA branch site. They also function at subsequent stages, for example, by facilitating the recruitment of U4/U6 and U5 snRNPs, which enter the spliceosome assembly pathway as a pre-formed U4/U6–U5 tri-snRNP particle. The RS domains of SR proteins are phosphorylated by several different kinases and this phosphorylation is important for the ability of the RS domains to interact with each other and for the activities of SR proteins in splicing (reviewed in Ref. 6). As will be discussed below, increasing evidence indicates that SR-family and SR-related proteins assemble on exonic splicing enhancer (ESE) sequences to promote both constitutive and regulated splicing by forming networks of interactions with each other, as well as with snRNP-associated, SR-related proteins.

Much of our initial understanding of ESE function has been derived from studies on the cascade of alternative splicing events that determine the sex of *Drosophila*. A pivotal step in this cascade is the regulation of inclusion of the female-specific exon 4 in the *doublesex* (*dsx*) gene (reviewed in Refs 3,7). This exon contains a specialized ESE consisting of six repeats of a 13-nucleotide consensus sequence and a purine-rich element located between repeats 5 and 6. A multisubunit complex that contains the SR-related, alternative splicing factors Tra (which is expressed only in females), Tra2 and SR-family proteins assembles on the *dsx* ESE. This complex facilitates the recognition of the immediately upstream, suboptimal 3′ splice site, thereby promoting exon 4 inclusion and, ultimately, the female differentiation pathway. Mammalian ESEs were identified initially as purine-rich sequences that, similar to the *dsx* ESE, associate with specific SR-family proteins and promote the utilization of adjacent splice sites (Ref. 11; reviewed in Refs 3,4).

The 'U2AF-recruitment' model for ESE function: evidence for and against

Protein–protein interaction screens have indicated that, through their RS

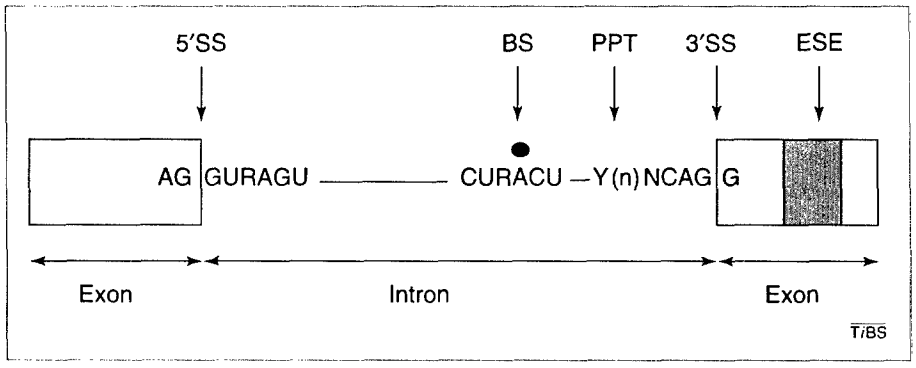

Figure 1
Pre-mRNA sequences important for splicing. Consensus mammalian 5′ splice site (5′ SS), branch site (BS; indicated by a black circle), polypyrimidine tract (PPT) and 3′ splice site (3′ SS) sequences are shown. ESE, exonic splicing enhancer; R, purine; Y, pyrimidine.

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domains, the SR-family proteins SC35 and ASF/SF2 can interact with each other, with the SR-related U1 snRNP-70kDa (U1-70K) protein, and with the small (35-kDa) subunit of the heterodimeric U2 snRNP auxiliary factor (U2AF-35/65kDa), which contains a short RS domain[8,9]. It had been demonstrated previously that the large subunit of U2AF (U2AF-65kDa), which contains three RRMs and also a short RS domain (Fig. 2), binds to the polypyrimidine tract located between the branch site and 3′ splice site and is required for the stable binding of U2 snRNP to the branch site (Ref. 10 and references within). These interaction studies led to the proposal that SR-family and SR-related proteins facilitate splicing by forming networks of interactions across introns and exons[8]. In the case of ESE function, it was proposed that SR proteins bound to ESEs promote splicing by facilitating the binding of U2AF-65kDa to the polypyrimidine tract through an interaction mediated by U2AF-35kDa (Fig. 3a). Support for this 'U2AF-recruitment' model comes from the observation that addition of SR proteins to splicing reactions resulted in increased crosslinking of U2AF-65kDa to pre-mRNA, although it was apparent in several cases that this increased crosslinking did not correlate with increased splicing of the pre-mRNA (e.g. Refs 11,12). Subsequently, using an assay consisting entirely of purified components, Maniatis and colleagues observed that crosslinking of U2AF-65kDa to an ESE-dependent pre-mRNA required U2AF-35kDa and a single SR-family protein, again suggesting a U2AF-recruitment model for ESE function[13].

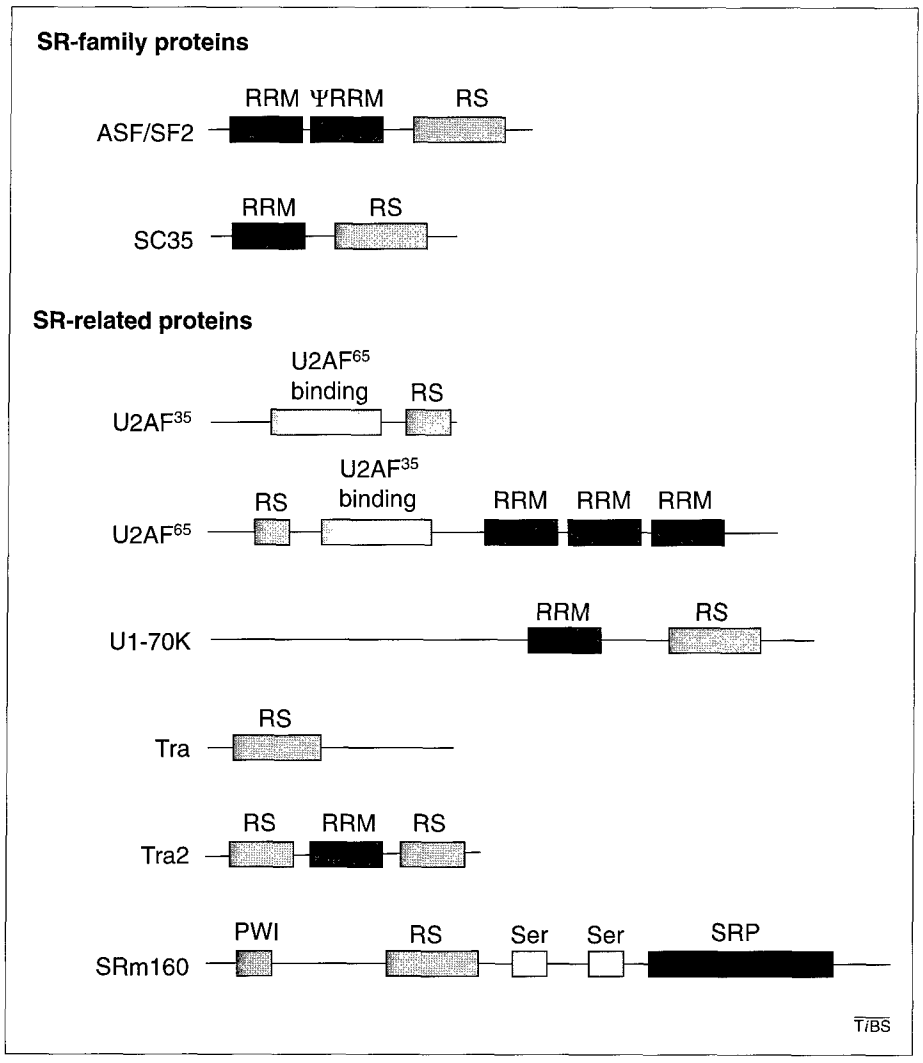

Figure 2

Domain structures of SR-family and SR-related proteins involved in pre-mRNA splicing. ASF/SF2 and SC35 are representative of a set of ten SR family proteins that contain one or two N-terminal RNA recognition motifs (RRM and a related ΨRRM motif) and a C-terminal domain that is rich in alternating serine and arginine residues (RS domain) (reviewed in Refs 3,4). SR-related proteins described in the text are depicted. Domains required for the interaction between U2AF-35kDa and U2AF-65kDa are shown. SRm160 contains several domains of unknown function including the PWI motif (PWI), which is shared with other splicing-related proteins[31], polyserine-rich motifs (Ser), and serine, arginine and proline-rich motifs (SRP)[21].

Contrary to the conclusions of these studies, more recent work has provided evidence that binding of U2AF-65kDa to ESE-dependent pre-mRNAs does not require interactions mediated by an ESE or U2AF-35kDa. In two independent studies employing splicing reactions containing total nuclear extract without exogenously added SR proteins, it was found that U2AF-65kDa bound equally to different pre-mRNAs in the presence and absence of an ESE (Refs 14,15). It was also found that a typical purine-rich mammalian ESE promoted the time-dependent association of several SR-family and SR-related proteins with the pre-mRNA, whereas U2AF-65kDa bound prior to most of these SR proteins, and its level of binding did not change significantly during the course of the reaction[15]. Moreover, it was observed that depletion of U2AF-35kDa did not affect the crosslinking of U2AF-65kDa to different ESE-dependent pre-mRNAs tested[14,16]. Interestingly, depletion of U2AF-35kDa has been observed to prevent the splicing of some ESE-dependent substrates, but not others[13,14,16]. The reasons for these different results are not clear, but could be attributed to differences between the specific substrates used or the method used to deplete U2AF-35kDa (immunodepletion versus biochemical depletion), or both. However, the emerging picture is that U2AF-35kDa, although important for the activity of specific substrates, appears not to be required for the binding of U2AF-65kDa to the polypyrimidine tract of several different ESE-dependent substrates. These findings are consistent with the recent observation that the RS domain of U2AF-35kDa is not required for viability or for the regulation of *dsx* pre-mRNA splicing in *Drosophila*[17].

The fundamental differences between the more recent experiments and the earlier ones, which suggested the U2AF-recruitment model, could be attributed to differences in the assays used. In particular, it is possible that the addition of exogenous SR proteins to reactions resulted in interactions that do not normally occur in splicing assays containing total nuclear extract without added SR proteins. SR proteins, at elevated concentrations, can form functionally redundant interactions. Thus, it is possible that the addition of exogenous

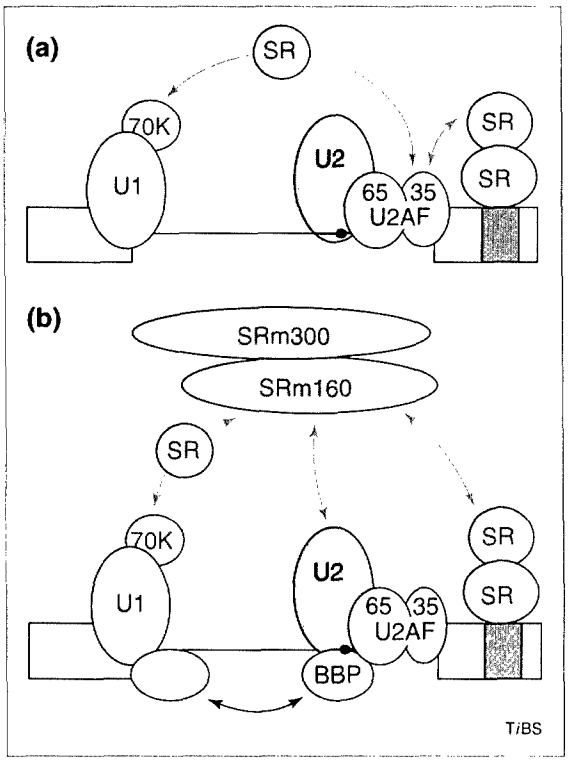

Figure 3

Models for exonic splicing enhancer (ESE) function. **(a)** The 'U2AF-recruitment' model for ESE function[8,32]. SR proteins bound to an ESE are proposed to stabilize the binding of U2AF-65kDa to the polypyrimidine tract through an interaction mediated by U2AF-35kDa. SR proteins are also thought to form interactions across the intron by binding to the U1 snRNP-specific 70kDa protein (70K) and U2AF-35kDa. **(b)** A new model for ESE function in which U1 snRNP promotes two sets of cross-intron interactions: one of which is ESE-independent (blue text on gray background), and the other is ESE-dependent (red text on yellow background). In contrast to the 'U2AF-recruitment' model, the ESE-independent interactions are sufficient for the stable binding of U2AF-65kDa to the polypyrimidine tract[14,15]. This set of interactions probably involves BBP (the branch-point-binding protein; also referred to as SF1 in mammals), which interacts with U2AF-65kDa, and the U1 snRNP-associated protein Prp40p (Ref. 25). These interactions are highly conserved between yeast (*S. cerevisiae*) and mammals, although Prp40p has been identified in *S. cerevisiae* but not in mammals; the light-blue lettering indicates the proposed existence of a similar protein in mammals (PRP40). The ESE-dependent interactions involve one or more SR proteins bound to an ESE, including SR-family and SR-related proteins (e.g. hTra2), that bridge to one or more snRNP components through a splicing coactivator such as SRm160/300 (Ref. 23). Both sets of U1 snRNP-dependent interactions are required for the stable binding of U2 snRNP to the branch site and the formation of a fully assembled spliceosome. Similar sets of interactions probably occur across exons, in accordance with the exon-definition model for splice-site selection (reviewed in Ref. 33). Different combinations of SR-family and SR-related proteins that function in conjunction with different ESEs are likely to play a major role in determining regulated patterns of splice-site selection in higher eukaryotes.

SR proteins to reactions promoted increased binding of U2AF-65kDa to pre-mRNA, although this increased binding might not reflect the mechanism by which ESEs function at endogenous concentrations of SR proteins. However, it should be borne in mind that the more recent findings do not exclude the possibility that a U2AF-recruitment mechanism could apply to specific ESE-dependent pre-mRNA substrates not analysed in these assays, or that such a mechanism could participate in ESE function *in vivo*, which also has not been investigated. In this regard, it is interesting to note that very recent reports indicate that U2AF-35kDa functions in the recognition of the conserved 3′ splice site AG sequence (see Fig. 1) and might stabilize the binding of U2AF-65kDa on specific substrates that are dependent on the 3′AG for the first step of splicing[18–20].

A role for the SRm160/300 splicing coactivator in ESE function

The recent experiments outlined above indicate that ESE-bound SR proteins can communicate with basal components of the spliceosome through a set of interactions that are separate from those required for the binding of U2AF-65kDa to the polypyrimidine tract. These interactions could be direct, involving contacts with one or more snRNP components, or indirect, through one or more factors that bridge ESE-bound SR proteins and spliceosome components, or both. A candidate for such a bridging factor has recently been identified: the SRm160/300 (complex consisting of two SR-related nuclear matrix proteins of 160-kDa and 300-kDa) splicing coactivator[21]. The SRm160/300 subunits are new SR-related proteins that, unlike members of the SR-family, lack RRMs (Refs 21,22). It has been demonstrated that SRm160/300 is required for a typical purine-rich mammalian ESE (consisting of six GAA repeats) to promote splicing of a pre-mRNA derived from exons 3 and 4 of the *dsx* gene (dsx pre-mRNA)[23]. The stable association of SRm160/300 with this pre-mRNA required the ESE and U1 snRNP. Moreover, its association with the substrate was mutually stabilized by U2 snRNP. Independent of pre-mRNA, SRm160/300 was found to interact specifically with a subpopulation of U2 snRNP and a subset of SR-family and SR-related proteins, including hTra2beta (Ref. 23), a human homolog of the *Drosophila* alternative splicing factor Tra2, which binds preferentially to ESEs containing GAA-repeats and activates ESE-dependent splicing (Ref. 24 and references within). These studies have led to a model for ESE function in which SRm160/300 promotes critical interactions between SR proteins, including SR-family proteins and hTra2beta bound to an ESE, and snRNP components of the spliceosome (Fig. 3b).

A more detailed analysis of factor interactions on the dsx pre-mRNA used in the above studies provided additional insights into the possible mechanism by which the stable binding of U2 snRNP to the branch site of an ESE-dependent substrate might be achieved. In addition to the association of SRm160/300, it was found that the stable binding of U2AF-65kDa to the dsx pre-mRNA also requires U1 snRNP, even in the presence of the ESE (Ref. 15). U1 snRNP, like U2AF-65kDa, binds equally to the dsx pre-mRNA in the presence and absence of the ESE (Ref. 23). Taken together, these findings indicate that, in the context of cross-intron interactions on an ESE-dependent substrate, U1 snRNP promotes two sets of interactions required for the stable binding of U2 snRNP to the branch site (see Fig. 3b): one set promotes the binding of U2AF-65kDa to the polypyrimidine tract and probably involves previously defined cross-intron interactions between U1 snRNP and U2AF-65kDa, involving the branch-point-binding protein BBP (also referred to as SF1 in mammals)[25]. Although required, this set of interactions is not sufficient for the stable binding of U2 snRNP. The second set involves SR-family and SR-related proteins, including SRm160/300, as described above. Because SRm160/300 was not found to interact with U1 snRNP in the absence of pre-mRNA, the model proposes the existence of a hypothetical SR protein that bridges between these components when U1 snRNP is bound to the pre-mRNA (Ref. 23). In summary, two sets of U1 snRNP-promoted interactions contribute to the formation of a productive splicing complex containing

Table 1. Human genetic disease mutations and ESE and ESS function

Disease	Protein product (function affected)	Location/ type of mutation	Splicing defect	Ref.[g]
Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)	Tau (microtubule-associated protein; promotes microtubule assembly and stability. Mutant Tau proteins form abnormal filamentous structures in the brains of FTDP-17 patients)[a]	Exon 10/missense[b] Exon 10/3-nt deletion[c] Exon 10/silent[d]	Exon 10 inclusion (constitutive) Exon 10 exclusion Exon 10 inclusion	34
Spinal muscular atrophy (SMA)	SMN1: survival motor neuron-1 (snRNP biogenesis)[a]	Exon 7/silent[e]	Exon 7 exclusion	36
Becker muscular dystrophy (BMD)	Dystrophin (muscle fiber formation)	Exon 27/nonsense[f]	Exon 27 exclusion	35

[a]Confirmation that the mutations in the Tau and SMN-1 genes affect exonic splicing enhancer (ESE) and exonic splicing silencer (ESS) sequences will also require evidence that these proposed elements are active when transferred to a heterologous context and are inactive when mutated.
[b]The missense mutation (N279K) results in constitutive exon-inclusion, probably by strengthening of the same ESE inactivated by Δ280K (Ref. 34).
[c]The 3-nt deletion in exon 10 of the tau gene (Δ280K) results in exon-skipping and is immediately 3′ to the missense mutation (N279K).
[d]The silent mutation (L284L), located 12 nt 3′ to the deletion mutation in exon 10, is proposed to affect an adjacent ESS element.
[e]A silent mutation in codon 280 within exon 7 of the SMN-1 gene results in exon 7 exclusion in a transfected mini-gene comprising exons 6–8, mimicking the splicing pattern of SMN-1 transcripts that contain this variance in SMA patients.
[f]The nonsense mutation in exon 27 of the dystrophin gene is located within a purine-rich element that functions as an ESE within the context of the *dsx* pre-mRNA (see text); the presence of the nonsense mutation abolishes enhancer activity[35].
[g]Other human genetic disease mutations that have been proposed to affect ESE and ESS function have been described in Refs 28 and 29.

U2 snRNP on an ESE-dependent substrate: one set is ESE-independent and is sufficient for U2AF-65kDa binding, and the other is ESE-dependent and involves SR-family and SR-related proteins.

Diversity and prevalence of ESEs: implications for human diseases

Besides efforts towards gaining mechanistic insights into how ESEs function, a major goal of recent studies has been to determine the precise nature of sequences that constitute an ESE. Surprisingly, 15–20% of sequences within a randomized 18- or 20-mer, when substituted for an ESE within different exon contexts, promote splicing (Refs 22,23 and references within). Functional SELEX (for 'systematic evolution of ligands by exponential enrichment') strategies employing randomized ESE-containing substrates have identified short (5–7 nucleotide) and highly degenerate consensus sequences that promote splicing in conjunction with single SR-family proteins[26,27]. In several cases, these consensus sequence(s) are very similar to the optimal consensus for binding to the SR protein, also obtained by SELEX. Sequences conforming to the SELEX consensus sequences are found within characterized ESEs and are more prevalent in exons than in introns of many genes that have been analysed[26,27]. These studies indicate that ESEs are comprised of a diverse spectrum of sequences that function in combination with different SR proteins. This flexibility in ESE composition is probably a major determinant underlying the coordination and regulation of pre-mRNA splicing patterns in higher eukaryotes. By analogy with mechanisms underlying cell-type- and differentiation-stage-specific transcriptional regulation that involve different transcriptional activator and coactivator proteins, it will be of interest to determine whether different ESEs that function by binding to different SR-family proteins require different splicing coactivators, such as SRm160/300. Alternatively, it is possible that coactivators, such as SRm160/300, serve a more general role by bridging different SR proteins bound to diverse ESEs.

The emerging prevalence of different ESE sequences in pre-mRNA exons has drawn attention to the possibility that these elements are frequent targets of mutations in human genetic diseases. Approximately 15% of point mutations that are linked to human genetic diseases cause splicing defects. Many of these mutations are located within exons, are separate from the splice junctions, and are not known to create cryptic splice sites (reviewed in Refs 28,29). In particular, an increasing number of reported disease mutations located in exons result in increased exon exclusion or inclusion (see Table 1 for recent examples). The mutations involved can be deletions, missense, nonsense or silent mutations and often affect a sequence that resembles a purine-rich ESE or one of the other degenerate consensus ESE sequences defined in the SELEX studies described above. In other cases, disease mutations have been identified that result in increased exon inclusion and have been proposed to affect exonic splicing silencer (ESS) sequences (Table 1). ESS sequences function to suppress splice-site selection and often appear to operate in conjunction with ESE sequences, which, when activated, are dominant over the adjacent ESS sequence (e.g. Ref. 14 and references within).

Given the widespread and critical importance of ESE sequences and the emerging importance of ESS sequences in the regulation of splice-site selection, it is perhaps not surprising that some diseases linked to aberrant splicing patterns do not appear to be due to *cis*-acting mutations, but instead appear to involve one or more (as yet unidentified) altered *trans*-acting factors. An important example is the deregulation of alternative splicing of pre-mRNA encoding the transmembrane glycoprotein CD44. CD44 normally functions in cell migration and attachment to the extracellular matrix and the surfaces of other cells. Spliced variants of CD44 have been detected in many different types of malignant tumors and have been correlated with poor patient survival in some studies[28]. Moreover, it has been demonstrated that expression of specific isoforms of CD44 containing alternative exons encoding extracellular domain sequences, confer full metastatic potential to isogenic non-metastatic cell lines[30]. It is possible that disease-associated changes in one or more factors required for normal ESE and ESS functions are responsible for the deregulation of alternative splicing events, such as those affecting the CD44 pre-mRNA. Gaining a deeper understanding of the factors involved in ESE and ESS function, and how the functions of these factors are altered in disease situations, undoubtedly will

contribute important and interesting insights into mechanisms underlying the normal and abnormal regulation of pre-mRNA splicing. These insights might lead to new strategies for the treatment of diseases in which aberrant splicing events play a causative role.

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